Molecular characterization of shattering in weedy rice (*Oryza sativa* f. *spontanea*) biotypes of Kerala

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Molecular characterization of shattering in weedy rice (*Oryza sativa* f. *spontanea*) biotypes of Kerala"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µM	Micromolar
A	Adenine
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
bp	Base pair
C	Cytosine
cDNA	Complementary DNA
cm	Centimetre
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
F	Forward primer
G	Guanine
g	Gram
g	standard acceleration due to gravity at earth's surface
h	Hour
kg	Kilogram
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
M-MuLV	Moloney murine leukaemia virus
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride

NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometre
nt	Nucleotide
°C	Degree celsius
OD	Optical density
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
R	Reverse primer
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT-PCR	Reverse transcription - polymerase chain reaction
s	Second
SH	Shattering
SD	Short day
sp.	Species
spp.	Species (plural)
t	Tonne
T	Thymine
TAE	Tris-acetate EDTA buffer
TE	Tris-EDTA buffer
T_m	Melting temperature
Tris HCl	Tris (hydroxy methyl) aminomethane hydrochloride
U	Enzyme unit
V	Volt
v/v	volume/volume
w/v	weight/volume

Introduction

1. INTRODUCTION

Rice is one of the major staple food crops in the world and is particularly significant in Asia where approximately 90 per cent of the world's rice is produced and consumed (Ziegler and Barclay, 2008). Improving the productivity of rice has achieved immense importance to feed nearly half of the world's population (Ratnasekera, 2015). One of the major constraints that obstruct these objectives is the inefficiency in management of problematic weeds such as 'weedy rice' in rice fields.

Weedy rice (*Oryza sativa* f. *spontanea*) evolved mostly by natural hybridization between wild and cultivated rice is an emerging threat to rice cultivation (Rathore *et al.*, 2013). It is a self-pollinated annual plant that is conspecific to cultivated rice (Xia *et al.*, 2011) and has became a menace infesting rice fields globally and most severely in Asia. In India an infestation level of 50-60 per cent has been reported from other states and yield reduction of 30-60 per cent has been documented in the rice fields of Kerala (Rathore *et al.*, 2016). It is not quite easy to differentiate weedy rice from cultivated ones during vegetative stages and by the time panicle emerges the damage is already done. With diverse biotypes, weedy rice has already infested large rice growing areas across the major rice tracts of Kerala and yield reduction to the tune of 30 to 60 per cent depending on severity of infestation like 3 to 10 mature plants of weedy rice per m^2 has been reported by Abraham *et al.* (2012).

The key characteristic of weeds, which subsidize to their success in agro ecosystems, is the genetic variability and plasticity found among and within weed populations (Green *et al.*, 2001). This makes the weeds to infest a wide range of diverse habitats. Genomics studies and research aim to enhance the awareness and understanding regarding the biological aspects of weed populations and enables us to predict and record the results of gene transfer among species (Weller *et al.*, 2001; Basu *et al.*, 2004). Through the modification of a suite of morphological and physiological attributes wild and weedy species were transformed into domesticated crops collectively referred to as domestication syndrome. The attribute or mechanisms to improve crop productivity and facilitate harvesting can be effectively studied through characterization of the genes related to the traits involved in it (Harlan, 1975).

In all cereal crops, the transition to domestication included a dramatic reduction in grain shattering (Harlan, 1992). However, important traits for the success of weedy rice are early shattering of the grain and variable seed dormancy (Azmi and Karim, 2008). While seed shattering is apparently a characteristic of all weedy rice, there is considerable variation in the time and degree of shattering among the phenotypes or ecotypes (Do Lago, 1982). On observing the extensive variability in shattering of weedy rice has made us reach the hypothesis that there are other genes connected to abscission layer integrity that might also be vital in regulating seed shattering in weedy rice. This, along with seed dormancy, contributes to the rapid propagation of weedy rice in infested fields. It results in an enriched seed bank which renders it difficult to manage. According to Delatorre (1999) In the absence of selective herbicides, managing shattering will help to reduce the weed seed bank. Hence, it is the need of the hour to address the present scenario with novel and innovative scientific methodologies involving molecular level interventions.

To understand the shattering process, it is essential to isolate and characterize the genes involved in shattering. Isolation and sequencing of genes related to shattering *viz. sh4* and *qsh1* in weedy rice biotypes and characterization by expression profiling and phylogenetic analysis would help in the development of an anti-shattering model which would help to lessen shattering and thereby reduce the weed seed bank. *sh4* (for grain shattering quantitative trait locus on chromosome 4) and *qsh1* (for quantitative trait locus of seed shattering on chromosome 1) genes have been identified as required for reduced seed shattering during rice (*Oryza sativa*) domestication. (Zhou *et al.,* 2012).

A comparison of the regulatory sequences of *sh4* and *qsh1*, the levels of gene expression, and the phenotypic difference among diverse weedy rice biotypes will shed meaningful light on the genetic basis of agricultural selection continued through the history of rice cultivation. This study can be repurposed for opening new avenues in rice genomic research and widening the possibilities of gene introgression in rice improvement. Studies on gene related to shattering in weedy rice may help to identify other genes which are linked with it and will help to relate their expression with various stages of the plant.

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In this backdrop, the present study was undertaken with the following objectives

- Isolation and sequencing of genes related to shattering viz. *sh4* and *qsh1* in weedy rice biotypes.
- Characterization of genes related to shattering by expression profiling and phylogenetic analysis.

Review of Literature

2. REVIEW OF LITERATURE

Rice (*Oryza sativa* L.) is one of the major staple food crops in the world and approximately 90 per cent of world's rice is produced and consumed in Asia (Zeigler and Barclay, 2008). Weedy rice is a complex of *Oryza* morphotypes of the grass family Poaceae with more than 20 wild species and 10 different genome types. Weedy rice (*Oryza sativa* f. *spontanea*), is taxonomically classified as the same species as cultivated rice (*O. sativa*), but is strongly characterized by its seed shattering which apparently increase the distribution of this species. The research works done on the molecular characterization of shattering genes in weedy rice in comparison with cultivated rice are reviewed in this chapter.

2.1 Weedy Rice

Weedy rice (*Oryza* f. *spontanea*), botanically classified as the same species as cultivated rice (Mortimer *et al.*, 2000) is one of the most notorious weeds in rice fields worldwide, causing both crop yield losses and degrading the quality of rice (Qiu *et al.*, 2014). It can be defined as any spontaneously and strongly shattering rice that occurs in cultivated rice fields which harbours phenotypes of both wild and domesticated rice. In regions of rice cultivation such as Asia and Latin America where native *Oryza* species occur, weedy rice may have arisen through a continual process of gene flow between the cultivated crop and neighbouring wild populations.

Weedy rice plant cannot be identified easily at seedling stage, as they are closer in appearance to cultivated rice. It generally grows faster with better use of the available N; produces comparatively more tillers, panicles and biomass, shatters early, has comparatively better resistance to adverse dry conditions, and possesses long and varied seed dormancy in soil. Morphologically seed and panicle characteristics of weedy rice are highly diverse and weedy rice plant is generally taller, profusely tillered, more open or spreading, have weaker culms, more susceptible to lodging, and exhibit more rapid seedling growth when compared to the cultivated rice to which they infest (Diarra *et al.*, 1985). Weedy rice produces fewer grains per plant and competes aggressively with the cultivated rice. High degrees of variations were observed among grain characteristics such as; presence

/ absence of awn, length and colour of the awn, hull and pericarp, grain size and shape, etc and among plant characteristics, such as number of panicles per hill, spikelets per panicle, percentage filled grains, grain weight etc. (Ratnasekera *et al.*, 2014).

Conspecific weeds like weedy rice are morphologically and ecologically different from cultivated and wild counterpart species. Studies indicate that in most cases hybridization between cultivated and wild species facilitates weed evolution, though they may evolve through genetic variation from either wild or cultivated species too (Reagon *et al.*, 2010). Mutations may also lead to evolution of conspecific weeds. Hence, weedy rice being genetically close and having a similar physiological life history to that of cultivated rice has created a vast challenge to rice farmers. He *et al.* (2014) claimed that rich genetic diversity permits weeds, including weedy rice, to adjust to an expansive scope of natural conditions, such as the change of habitat temperature, change of farming styles, and aggravation, causing problems for agriculture production.

The seeds of most weedy biotypes of *O. sativa* and *O. glaberrima* have a pigmented pericarp resulting from the presence of a variable content of different anthocyanins, cathekins and cathekolic tannins (Baldi, 1971). The red pigmentation is a dominant character and is controlled by more than one gene (Wirjahardja *et al.*, 1983). Weedy rice grains frequently have a red pigmented pericarp and it is for this reason that the term 'red rice' is commonly adopted in international literature to identify these wild plants. This term, however, does not seem very appropriate as red-coat grains are also present in some cultivated varieties, but also absent in various weedy forms (FAO, 1999).

Application of the tools of genomics has been found to be useful in understanding the processes of both crop evolution and crop improvement (Paterson, 2002). Because of its similarity to cultivated rice variety and abundance of genomic resources including the whole genome sequences (IRGSP, 2005), weedy red rice stands as an ideal experimental organism to investigate the genetic mechanisms of evolution in red rice and the molecular basis of weedy characteristics.

2.2 Origin of weedy rice

According to Chen *et al.* (2004), wild rice species, particularly those of the *O. sativa* (AA) complex (i.e., *O. rufipogon*, *O. barthii* and *O. longistaminata*) and three species of the *O. officinalis* complex (i.e., *O. punctata*, *O. latifolia* and *O. officinalis*), have become invasive and troublesome weeds in rice production system. They are expected to be the progenitor of weedy rice. Weedy rice exhibit both phenotypic and genotypic variations (Kumar and Ladha, 2011) and such variations lead to the evolution of weedy rice morphotypes and ecotypes.

The phylogenetic starting point of the weedy structures is firmly identified with that of cultivated rice. Numerous weedy plants share the vast majority of the highlights of the two cultivated species *Oryza sativa* and *O. glaberrima* (Khush, 1997). *O. sativa*, which is otherwise called Asian, includes the varietal bunches *indica, japonica* and *javanica*, and is become around the world (Olofsdotter, 1999). *O. glaberrima* is likewise named African rice and is mostly developed in West Africa. The family Oryza incorporates more than 20 wild species the vast majority of which are diploid. In light of the morphological, physiological, biochemical highlights and intersection connections, eight unique genomes have been distinguished in the variety Oryza (Aggarval *et al.,* 1997). Weedy rice found in regions where no wild rice occurs, is probably the derivatives of cultigens. They have naturally being selected for weediness either from cultigens or from progeny of natural hybridization between different cultivars. Such weedy plants may have been established for a long time at low frequency with higher adaptability than the improved cultivars when and where adverse conditions prevailed (Roy, 1921).

Since India is considered as the center of origin of cultivated rice, germplasm of various weedy and wild relatives are associated with this rice growing area. Similar to two groups of rice cultivars *viz*., *indica* and *japonica*, weedy rice is also classified into two and further classification was possible based on the cultivated and wild types. According to Suh *et al.* (1997), Indian weedy rice belongs to *indica* type, emphasizing that these weedy rice biotypes might have originated by natural hybridization between cultivated rice (*indica* type) and wild rice.

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Federici *et al.* (2001) carried out a study on 26 Uruguayan weedy accessions and classified them into two main groups of samples. One group included plants with a black hull, purple apex and long awn, showing evident wild traits, while the other group had straw hull and apex and no awn mimicking cultivated varieties. Weedy plants show a wide variability of anatomical, biological and physiological features (Vaughan *et al.* 2001).

Suh *et al.* (1997) reported that weedy rice has been classified into two groups corresponding to the *indica* and *japonica* sub species through genetic characterization of weedy rice accessions associated with *O. sativa*. Further, in both groups, two types with different propagating systems have been recognized; one is a crop mimic type that is unconsciously seeds and harvested by humans mixed with cultigens, and the other is naturally propagating type, which disperse its seeds, although the variation between the two types is continuous.

The continued presence of weedy rice in a rice field is attributed, in part, to its ability of strong seed shattering at maturity and seed dormancy, which can promote the persistence of weedy rice seeds in soil seed-banks (Cohn and Hughes, 1981). Weedy rice in rice fields is characterized by its easy seed shattering, deep dormancy and red pericarp which serve as key to distinguish weedy rice from wild species and cultivated rice (Gealy *et al.*, 2000). Some non-dormant weedy rice populations have evolved into new mechanism for respond to a critical habitat temperature for seed germination (Xia *et al.*, 2011).

Hypothetically, weedy rice is evolved either from the natural hybridization between rice cultivars and wild rice relatives (exo-ferality) (Reagon *et al.*, 2010), or from cultivated progenitors directly through the de-domestication process (endo-ferality) (Xia *et al.*, 2011).

2.3 Weedy rice accessions and cultivated rice

Subasinghe *et al.* (2007) molecularly characterized weedy rice in Sri Lanka. This study was undertaken to use morphological as well as molecular techniques to identify weedy rice collected from cultivated paddy fields, in an attempt to elucidate

the relationship of these weedy forms with the cultivated and wild rice varieties. The two weedy rice accessions (Weedy Rice-1; collected from Puttlam district, north western province) and Weedy Rice-2 (collected from Galle district, southern province) were subjected to Random Amplified Polymorphic DNA (RAPD) analysis together with a popular new improved rice variety Bg-300, a traditional variety (*Kaluheneti*), and an annual wild type (*O. nivara*). There morphological developments were also studied in plant house conditions. Among 11 primers used in the DNA fingerprinting, 6 primers (OPF-4, OPF-16, OPF-18, OPM-10, OPM-18, OPY-11) showed specific bands that helped to differentiate the samples tested. The dendrograms obtained from both molecular and morphological analysis clearly separated the cultivated varieties, wild rice and weedy rice into different clusters. The shortest genetic distance was observed between Bg-300 and *Kaluheneti* while the highest genetic distance as observed between *O. nivara* and the weedy rice accessions used in the study. The morphological analysis indicated that weedy rice show intermediate characteristics to those of cultivated and wild (annual) rice. In both analyses, the positioning of two weedy rice accessions between the cultivated and annual wild type (*O. nivara*) indicated the potential of the occurrence of a hybrid between wild and cultivated rice.

2.4 Seed Shattering in weedy rice

Seed shattering is an adaptive trait for seed dispersal in wild plants. However, the seed shattering habit causes yield loss for domesticated crop plants during harvest. Yearly addition of weedy rice seeds to soil seed bank intensifies the problem over years and management of weedy rice becomes more difficult (Chauhan, 2013).

Seed shattering occurs in the anatomically distinct cell files known as the abscission zone (AZ). Differentiated AZ cells are small, isodiametric, and cytoplasmically dense compared with surrounding cells and are responsive to signals promoting abscission (McKim *et al.*, 2008). Our ancestors began domesticating crop plants by selecting grains that had reduced seed shattering characteristics (Fuller *et al.*, 2009). Often these signals are associated with the senescence of the distal organ. However, a spectrum of environmental factors, such as a deficit or surplus of water,

extremes of temperature, or pest and pathogen attack, can prematurely precipitate leaf, flower, or fruit fall (Taghizadeh *et al.*, 2009). Understanding how the process of abscission is regulated in model crops would benefit agriculture.

Early seed shattering is a specific characteristic of weedy rice. The seed drop results from a formation of an abscission tissue formed by three layers of cells between the spikelet and the pedicel (Nagao and Takahashi, 1963). This layer of cells is not fully formed in cultivated varieties and bands of lignified tissue provide the bind of the spikelet to the pedicel.

The analysis of the evolutionary genetic variations related to the domestication process provides new perspectives about human interventions as well as vistas for crop improvement. Limited seed shattering (SH) is a typical domestication trait that separates crop cultivars from their wild ancestors. Heavy shattering, which has been removed during the domestication process, is a significant adaptive feature for effective expansion and persistence of all wild and weedy species. The degree of seed shattering in rice cultivars is dependent on the methods of harvest followed in different geographic regions, even though a moderate degree of seed shattering is usually allowed in cultivated species (Warwick and Stewart, 2005).

Hand and combine harvesting can be done using moderate shattering varieties; however, hard-to-thresh or non shattering varieties are required for harvesting by small headfeeding combines. In order to reduce crop loss, shattering is routinely evaluated in breeding methods. A precise localization of SH loci will have a positive impact upon rice breeding programs. This is so because progress in exploiting wild rice species for improvement of rice crop is delayed or restricted due to the linkage of seed shattering genes with desirable features or traits (Kobayashi, 1990).

2.5 Genes controlling shattering in weedy rice

Shattering behaviour is controlled by the gene *sh* which shows the shattering character in conditions of dominant homozygosys (*ShSh*) or heterozygosys (*shSh*) (Sastry and Seetharaman, 1973). Molecular level works on the shattering ability showed that abscission layer was formed by the inactivation of the CTD phosphates like gene *OsCPL 1* hastening seed shattering in rice (Abraham *et al.*, 2012).

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sh4 is considered the most significant shattering gene to have been selected upon during domestication (Li *et al.*, 2006; Purugganan and Fuller, 2009). Examination of *sh4* alleles has shown that all cultivated rice sampled to date shares the non-shattering T mutation, and most rice individuals share a common *sh4* haplotype, despite the fact that at least two separate domestication events gave rise to cultivated Asian rice. The sharing of a common *sh4* haplotype across divergent rice varieties has been attributed to a combination of introgression and strong positive selection (selective sweep) favouring a reduction in shattering in the crop during both domestication processes (Zhang *et al.*, 2009).

The shattering allele of the *sh4* gene is replaced by the mutant non-shattering allele, and this is generally seen as a significant event in the evolution of cultivated rice. However, this claim has been challenged by many researchers when more diverse wild, weedy, and cultivated rice samples were taken to analysis (Zhu *et al.*, 2012). This might also be the case of sharing of similar haplotypes of *sh4* associated with reduced seed shattering in both weedy and cultivated rice varieties.

A wide range of seed shattering can be seen in cultivated rice. *Indica* varieties are generally found to be more easily shattering than *japonica* varieties. By using the classical genetics approach, four shattering genes viz., *sh1*, *sh2*, *sh3* and *sh4* were identified and then localized on chromosomes 11, 1, 4 and 3 respectively in rice. When a number of quantitative trait locus (QTL) mapping analysis was done in wild species (*Oryza rufipogon* (Ishikawa *et al.*, 2010), *Oryza nivara* (Li *et al.*, 2006a), weedy rice (Thurber *et al.*, 2013), and cultivated rice (Qin *et al.*, 2010)), complex polygenic control for SH was revealed.

The molecular basis of SH and the domestication process were unraveled through cloning of four shattering loci, *sh4, qsh1, shat1,* and *sh-h*. The formation of the abscission layer between the grain and pedicel is found to regulate the seed shattering in rice. A transcription factor with a *Myb3* DNA binding domain and nuclear localizing signal is encoded in the *sh4* gene was isolated from the wild species *O. nivara* (Li *et al.*, 2006b). Cai and Morishima (2000) observed that a single nucleotide substitution of G to T at the *sh4* locus is the reason for reduced

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SH in cultivated rice. A distinguishing feature of weedy red rice is the red pericarp (*Rc*) which is believed to be closely connected with SH and is eliminated during the process of rice domestication (Sweeney *et al.*, 2007).

The QTL *qsh1* was identified by Konishi *et al.* (2006) through analysis of a population developed from a cross between a shattering *indica* type, Kasalath, and a non shattering *japonica* cultivar called Nipponbare. The study revealed that the reason for reduced SH in *japonica* rice cultivars is due to single nucleotide polymorphism (SNP) in the 5′ regulatory region of a BEL1-type homeobox gene.

A nuclear phosphatase, that prevents the formation of the abscission layer, is encoded in the *sh-h* gene, which is cloned from a shattering mutant gene (Ji *et al.*, 2010). There is a wide range in the level of seed shattering among overall rice cultivars (Konishi *et al.*, 2006), that the shattering propensity is a polygenic and complex characteristic. To better comprehend the procedure of seed abscission and endeavor the system of various qualities managing the rice shattering pathway, Zhou *et al.*, 2012 investigated suppress or mutants in a genetic background containing known seed shattering–related genes in which the shattering events have in some way been impaired. To this end, they constructed a shattering chromosomal segment substitution line (CSSL), Substitution Line 4 (SL4), by introducing chromosome 4 of wild-rice *Oryza rufipogon* W1943 (easy shattering) into the recurrent parent *O. sativa* ssp *indica* cv Guangluai 4 (GLA4) (reduced shattering). They identified a number of new non-shattering rice mutants by 60 Co g-ray mutagenesis of SL4.

To investigate the molecular regulation of seed shattering, Zhou *et al.* (2012) mutagenized an easy shattering line with an introgression of a chromosome segment with the *sh4* locus from *O. rufipogon* in order to investigate the molecular controlling of seed shattering. Two non-shattering mutants were also identified in this process. Through the map based cloning approach they identified an *ap2* domain containing the transcription factor gene, *shat1*, in one mutant, that affects the development of the abscission zone. A null allele of *sh4* with a stronger non-shattering phenotype was the other mutant.

2.6 Molecular characterization of seed shattering gene

Previous studies of rice (*Oryza sativa*) have identified a few of the factors involved in seed shattering. The cultivated rice allele of *sh4* severely weakens but does not eliminate shattering (Li *et al.*, 2006a). *sh4* is a member of the trihelix family of transcription factors and promotes hydrolyzing of AZ cells during the abscission process (Lin *et al.*, 2007). *qsh1*, a major rice quantitative trait locus on chromosome 1, encodes a BEL1-type homeobox-containing protein.

A single nucleotide polymorphism (SNP) in the 59 upstream regulatory region of *qsh1* causes *qsh1* expression to disappear from the abscission layer, thus leading to a decline in seed shattering over the history of rice domestication (Konishi *et al.*, 2006). According to Simons *et al.* (2006), in wheat (*Triticum turgidum*) *Q* gene was reported to affect the compaction and fragility of wheat ears and also the ease with which the grain can be separated from the chaff. *Q* is a member of the *apetala2* (*ap2*) family of transcription factors, which have been implicated in a wide variety of plant development roles. Recently, a recessive shattering locus *sh-h*, encoding a C-terminal domain phosphatase- like protein, was identified using mutagenesis of cultivated rice and was shown to inhibit the development of AZs in rice (Ji *et al.*, 2006).

Thurber *et al.* (2010) came to the conclusion that to reduce SH a single nucleotide mutation in the *sh4* gene alone will not be sufficient. Likewise, Zhu *et al.* (2012) observed the presence of the non-shattering *sh4* allele in all weedy rice types as well as in wild species with shattering phenotype in high level frequency. In addition, they also made a hypothesis that there might be other unidentified shattering loci that played vital role in the initial domestication of cultivated rice.

However, the involement of multiple loci in the rice domestication process was suggested by Izawa (2008). Thus, in order to understand the rice domestication process completely, we need QTL mapping studies of diverse materials

Simple sequence repeats (SSRs) or microsatellites are stretches of DNA, consisting of tandemly repeating mono -, di -, tri -, tetra - or penta - nucleotide units that are widely distributed across all eukaryotic genomes (Powell *et al.*,1996). In

addition, the uniqueness and value of microsatellites arises from their multiallelic nature, codominant transmission, and ease of detection by PCR. Mc Couch *et al.* (2002). has developed a total of 2414 new di-, tri- and tetra-nucleotide non-redundant SSR primer pairs, representing 2240 unique marker loci, which are experimentally validated for rice (*Oryza sativa* L.). The utility of these microsatellites lies in their high information content (i.e., the number and frequency of alleles detected); and ease of genotyping. The excellent attributes of DNA markers and the microsatellite markers in rice make it possible for better understanding of weeds, including the population biology and development of novel approaches to weed management. However, DNA markers were also applied to evaluate genetic diversity of plant germplasm (Keivani *et al.*, 2010).

Prathepha (2011) evaluated the genetic diversity of a collection of weedy rice from Thailand Laos. By way of the microsatellite analysis of weedy rice (*Oryza sativa* f. *spontanea*). Ninety-nine weedy rice accessions from four populations were evaluated by means of four SSR markers. A total of 49 alleles were detected. The number of alleles per locus ranged from 3.6 to 8.7, with an average of 7.6. The overall genetic diversity of weedy rice populations was relative high (*Hs*=0.619). The genetic differentiation among the four populations showed that genetic variability mainly existed among weedy rice individuals rather than among populations in all four populations. Results from the present study reveals a clearer understanding of the genetic diversity of the weedy rice in Thailand and Laos.

Subudhi *et al.* (2013) described the mapping of seed shattering loci providing insights into origin of weedy rice and rice domestication. The genetics of seed shattering was investigated in this study to provide insights into rice domestication and the evolution of weedy rice. Quantitative trait locus (QTL) analysis, conducted in 2 recombinant inbred populations involving 2 rice cultivars and a weedy rice accession of the southern United States, revealed 3–5 QTLs that controlled seed shattering with 38–45% of the total phenotypic variation. Two QTLs on chromosomes 4 and 10 were consistent in both populations. Both cultivar and weedy rice contributed alleles for increased seed shattering. Genetic backgrounds affected

both QTL number and the magnitude of QTL effects. The major QTL *qsh4* and a minor QTL *qsh3* were validated in near - isogenic lines, with the former conferring a significantly higher degree of seed shattering than the latter. Although the major QTL *qsh4* overlapped with the *sh4*, the presence of the non-shattering single nucleotide polymorphism allele in the weedy rice accession suggested involvement of a linked locus or an alternative molecular genetic mechanism. Overlapping of several QTLs with those from earlier studies indicated that weedy rice may have been derived from the wild species *Oryza rufipogon*. Natural hybridization of rice cultivars with the highly variable *O. rufipogon* present in different geographic regions might be responsible for the evolution of a wide range of phenotypic and genotypic variabilities seen in weedy rice populations worldwide.

He *et al.* (2017) identified the origin and signatures of a selection of Korean weedy rice by population genomics. In this study, they investigated the phylogenetics, population structure and signatures of a selection of Korean weedy rice by determining the whole genomes of 30 weedy rice, 30 landrace rice and ten wild rice samples. The phylogenetic tree and results of ancestry inference study clearly showed that the genetic distance of Korean weedy rice was far from the wild rice and near with cultivated rice. Furthermore, 537 genes showed evidence of recent positive or divergent selection, consistent with some adaptive traits. This study indicates that Korean weedy rice originated from hybridization of modern *indica/indica* or *japonica/japonica* rather than wild rice. Moreover, weedy rice is not only a notorious weed in rice fields, but also contains many untapped valuable traits or haplotypes that may be a useful genetic resource for improving cultivated rice.

In the study of selection on grain shattering genes and rates of rice domestication, Zhang *et al.* (2009) studied nucleotide variation at the shattering loci, *sh4* and *qsh1*, for cultivated rice, *Oryza sativa* ssp. *indica* and *Oryza sativa* ssp. *japonica*, and the wild progenitors, *Oryza nivara* and *Oryza rufipogon*. The non shattering *sh4* allele was fixed in all rice cultivars, with levels of sequence polymorphism significantly reduced in both *indica* and *japonica* cultivars relative to the wild progenitors. The *sh4* phylogeny together with the neutrality tests and

coalescent simulations suggested that *sh4* had a single origin and was fixed by artificial selection during the domestication of rice. Selection on *qsh1* was not detected in *indica* and remained unclear in *japonica*. Selection on *sh4* could be strong enough to have driven its fixation in a population of cultivated rice within a period of approx. 100 years. The slow fixation of the non-shattering phenotype observed at the archeological sites might be a result of relatively weak selection on mutations other than *sh4* in early rice cultivation. The fixation of *sh4* could have been achieved later through strong selection for the optimal phenotype.

Genetic control of seed shattering in rice was disclosed by Zhou *et al.* (2012) using the *apetala2* transcription factor shattering abortion1. They identified a seed shattering abortion1 (*shat1*) mutant in a wild rice introgression line. The *shat1* gene, which encodes an *apetala2* transcription factor, is required for seed shattering through specifying abscission zone (AZ) development in rice. Genetic analyses revealed that the expression of *shat1* in AZ was positively regulated by the trihelix transcription factor *sh4*. They also identified a frameshift mutant of *sh4* that completely eliminated AZs and showed non-shattering. These results suggest a genetic model in which the persistent and concentrated expression of active *shat1* and *sh4* in the AZ during early spikelet developmental stages is required for conferring AZ identification. *qsh1* functioned downstream of *shat1* and *sh4*, through maintaining *shat1* and *sh4* expression in AZ, thus promoting AZ differentiation.

From the viewpoint of weedy rice management, it is preferable to identify genetic diversity. Appropriate management of weeds occurring in agro-ecosystems will harmonize the systems and enhance the sustainable crop production. A central characteristic of weeds, which contributes to their success in agro-ecosystems, is the genetic variability and plasticity found within and among weed populations (Green *et al.*, 2001). This enables weeds to infest a wide range of diverse habitats. Thus, a full understanding of the genetic diversity of weeds is a major prerequisite for their effective management. In addition, elucidating the origin and evolutionary processes of weeds is helpful for designing effective management strategies for weed control (Pysek and Prach, 2003).

Materials and Methods

3. MATERIALS AND METHODS

The study entitled 'Molecular characterization of shattering in weedy rice (*Oryza sativa* f. *spontanea*) biotypes of Kerala' was conducted at the Department of Plant Biotechnology and Department of Agronomy, College of Agriculture, Vellayani, Thiruvananthapuram, during 2015-2017. This chapter describes the experimental materials used and the methodology followed for various experiments.

3.1 PLANT SAMPLE COLLECTION

The severity of weedy rice infestation in rice and the morphological diversity of weedy rice biotypes present in the rice tracts of Kerala is presented in plate 1, 2, 3 and 4. Seeds of the most common weedy rice (*Oryza sativa* f. *spontanea*) biotype were collected from the infested rice fields of Kanjirathadi padashekharam. *Uma* (MO 16) seeds procured from Regional Agricultural Research Station (RARS), Pattambi were used for raising the plants in pots at Department of Agronomy, College of Agriculture, Vellayani. Leaf and culm samples were taken at respective growth stages *viz*., seedling stage (15 Days After Sowing (DAS), and tillering stage (30-45 DAS). Flag leaf and panicles were taken at flowering stage. Each sample was properly labelled and then packed in a polypropylene bag. After chilling in liquid nitrogen, the samples for RNA and DNA isolation were stored at -80°C (Panasonic MDF U55V-PE), for further downstream processing.

3.2 IDENTIFICATION OF GENES RELATED TO SHATTERING

For isolation and identification of genes related to shattering *viz. sh4* and *qsh1* in weedy rice, degenerate primers were designed on the basis of the sequences of the genes reported to regulate shattering in several other rice species. These primers were analyzed for amplification by PCR and RT-PCR in genomic DNA and mRNA in the most common weedy rice biotype and cultivated rice variety *Uma*.

3.2.1 Degenerate Primer Designing

Though several genes are reported to regulate seed shattering in various *Oryza* species, *sh4* and *qsh1* are reported as more significant. So nucleotide sequences of *sh4* gene and *qsh1* gene from several rice species *viz., Oryza sativa, Oryza rufipogon,*

Plate 1. Weedy rice (*Orysa sativa* f. *spontanea*)

Plate 2. Difference in plant height between *Uma* & weedy rice

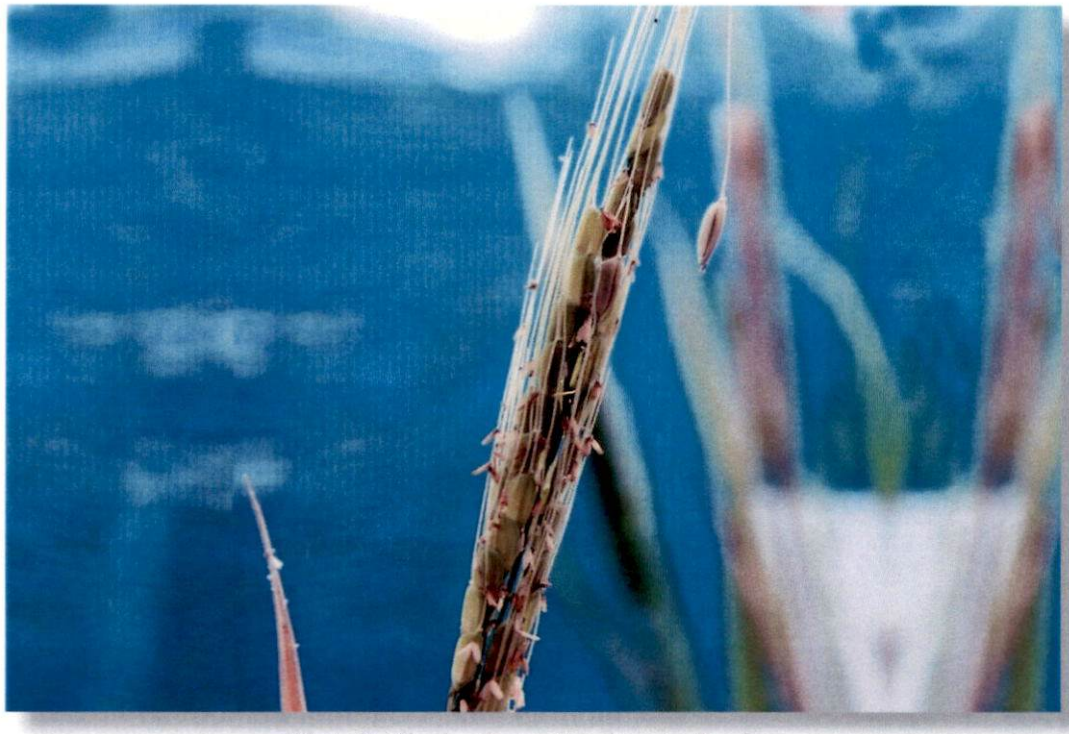


Plate 3. Grain shattering in weedy rice

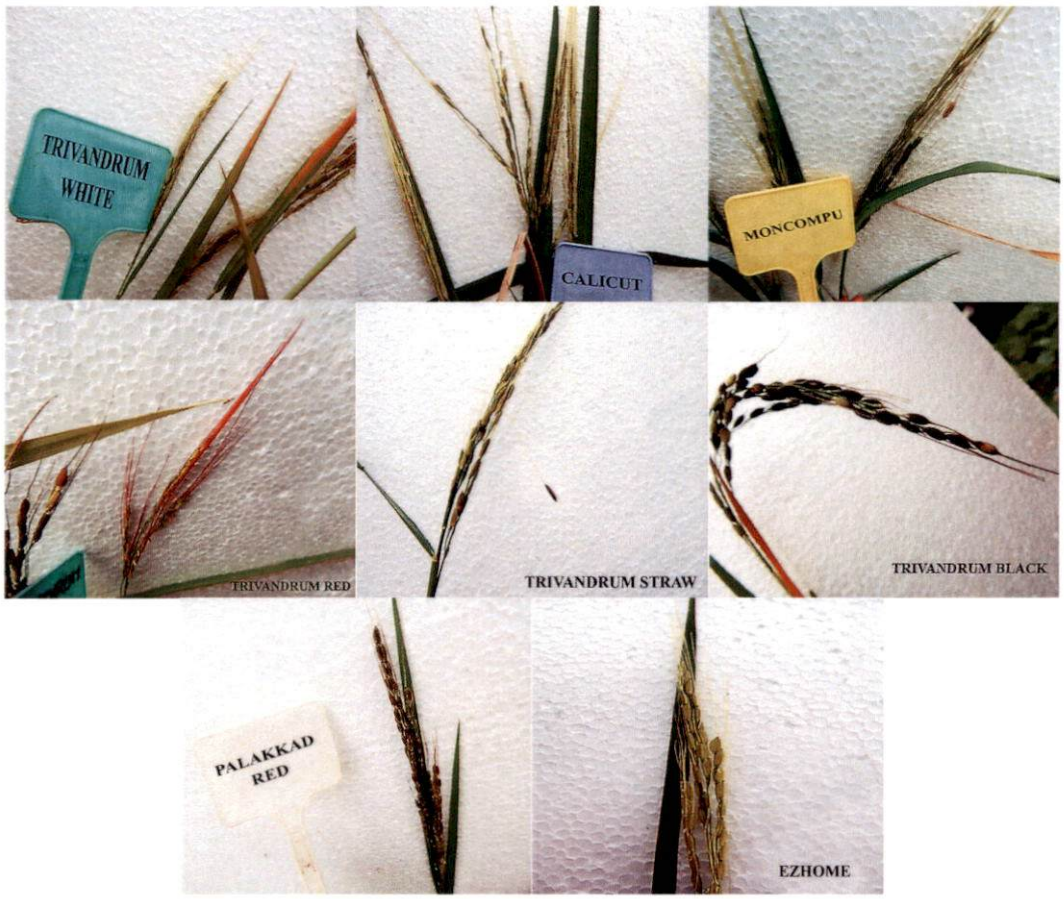


Plate 4. Variation in awn colour of weedy rice biotypes from different places of Kerala

Oryza nivara, *Oryza meridionalis*, *Oryza sativa Indica*, and *Oryza sativa Japonica* were retrieved from NCBI (National Centre for Biotechnology Information), GenBank and European Nucleotide Archive (ENA) database and downloaded in FASTA format and multiple aligned using Clustal X software (Thompson *et al.*, 1997). Later degenerate primers were designed for genes *viz.*, *sh4* and *qsh1* using bioinformatic tools such as Clustal X and Sequence Manipulation Suite. (Fig. 1 and 2).

3.2.1.1 Primer Analysis

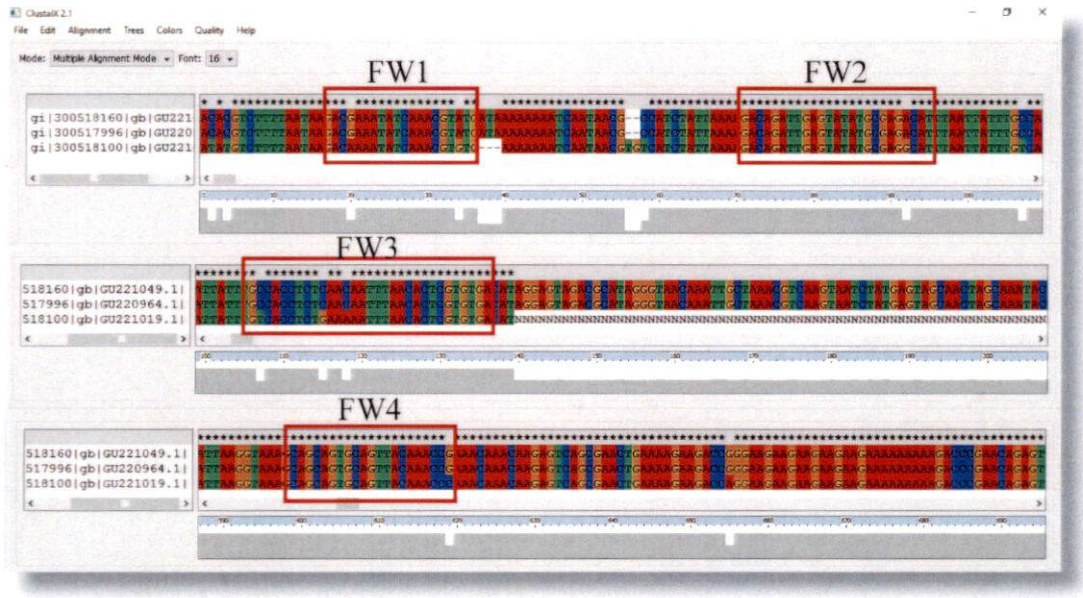
The designed set of primers were checked for several parameters such as primer length, product length, low degeneracy, maximum specificity at the 3' end, before their synthesis. The properties such as annealing temperature, GC-content, potential hairpin formation and 3' complementarity were analyzed by using Oligo Calc program (http://simgene.com/OligoCalc). The sequences of the resultant primers were sent to 'Sigma Aldrich' for synthesis.

3.2.2 Genomic DNA Isolation

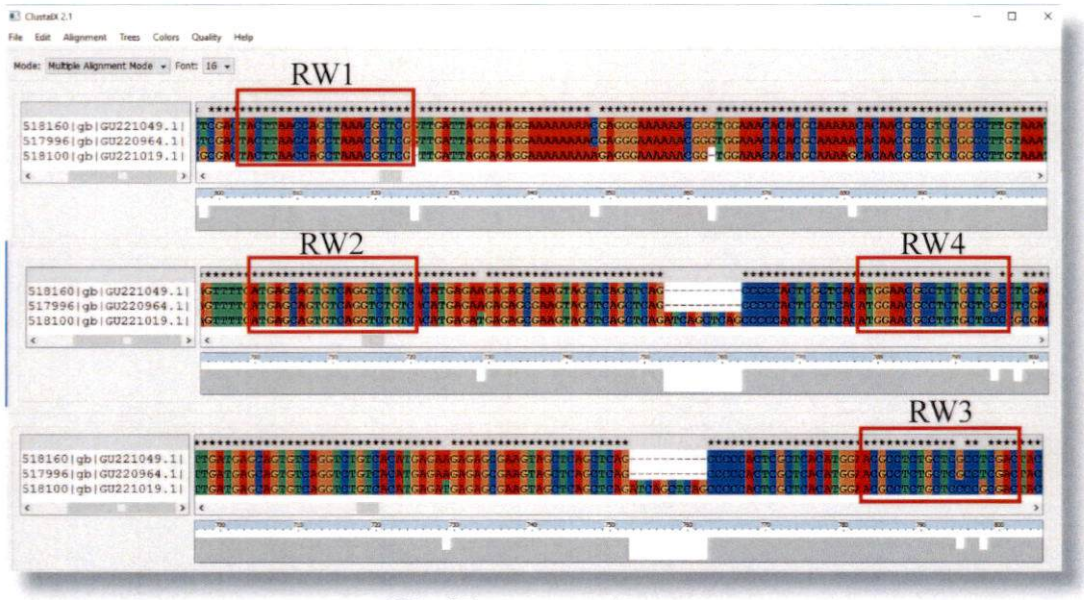
Genomic DNA was isolated from both weedy rice and cultivated rice variety *Uma* from flag leaf utilizing modified SDS method of genomic DNA standardized by Dellaporta *et al.* (1983). Modified CTAB method developed by Liang *et al.* (2015) was followed for DNA isolation from panicles at flowering stage.

3.2.2.1 SDS method of Genomic DNA Isolation

20µl of β-mercaptoethanol and 50 mg of PVP (Polyvinyl pyrolidone) were added to 15 ml of Dellaporta extraction buffer (Appendix I) in sterile 15 ml Falcon tubes and and kept at 4°C. Three grams of samples were chilled and ground into a fine powder in liquid nitrogen using a sterile pre-chilled mortar and pestle. The powdered sample was transferred into15 ml of extraction buffer kept at 4°C and kept on ice. To the mixture 1ml of 20 per cent SDS was added, the content was homogenized by gentle inversion, and incubated at 65°C for 1 hour in waterbath (RWB-01, ROTEK, India) with intermittent shaking. To the homogenate 5ml of 5*M* potassium acetate was then added and kept on ice for 20 min. Centrifugation



(A) *sh4* forward primers



(B) *sh4* reverse primers

(*) - Conserved nucleotide

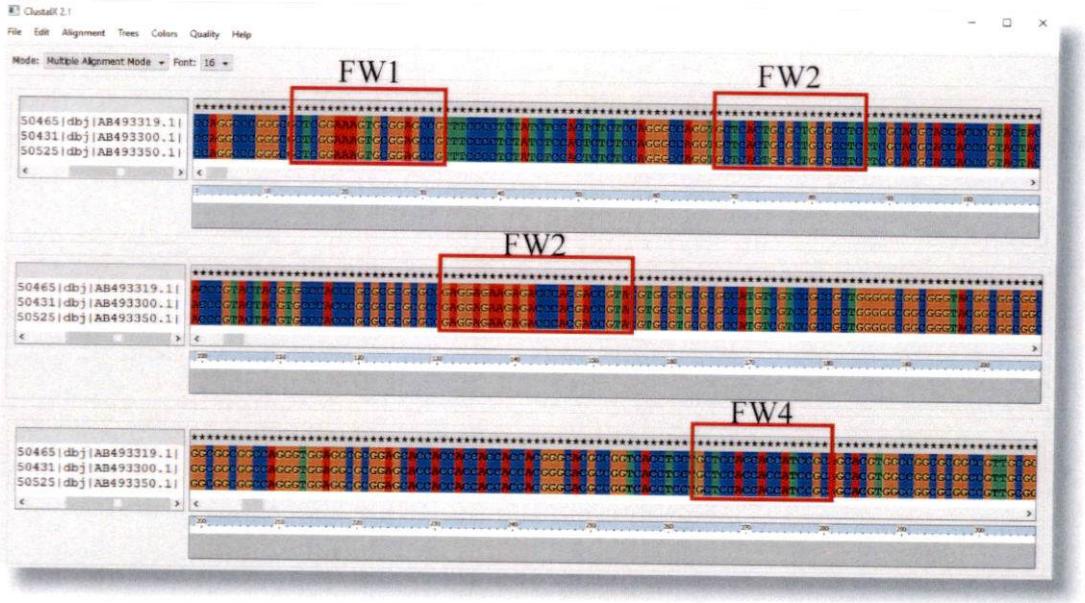
[ ] - Region used for primer designing

FW - Forward primer name

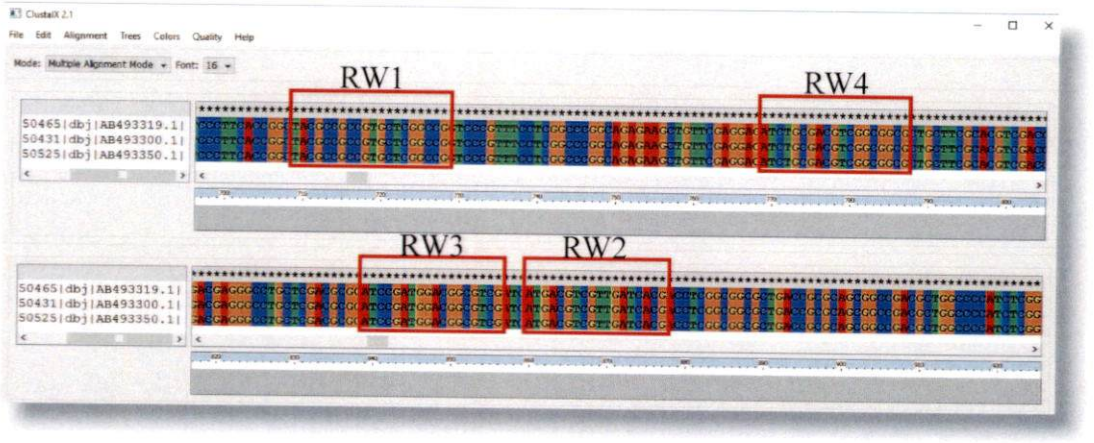
RW - Reverse primer name

Accession Number	Plant Species
GU221049.1	*Oryza rufipogon*
GU220964.1	*Oryza sativa*
GU221019.1	*Oryza meridionalis voucher*

Figure 1. Multiple sequence alignment for designing degenerate *sh4* primer: (A) *sh4* forward primers, (B) *sh4* reverse primers



(A) *qsh1* forward primers



(B) *qsh1* reverse primers

(*) - Conserved nucleotide

[ ] - Region used for primer designing

FW - Forward primer name

RW - Reverse primer name

Accession Number	Plant Species
AB493319.1	*Oryza sativa indica* group
AB493300.1	*Oryza sativa japonica* group
AB493350.1	*Oryza rufipogon*

Figure 2. Multiple sequence alignment for designing degenerate *qsh1* primer: (A) *qsh1* forward primers, (B) *qsh1* reverse primers

(Hermle Centrifuge Z 326 K, Germany) was performed at 12,000 rpm for 20 min. and the clear aqueous phase was transferred to a new sterile tube. Equal volume of ice cold isopropanol was added and mixed gently by inversion and then kept in the freezer (-20°C) until DNA was precipitated out. Centrifugation was performed at 12,000 rpm for 10 min. and the pellet obtained was dissolved in 500µl sterile double distilled water. This DNA solution was transfered to eppendorf tube and then 3µl of RNase was added and incubated at 37°C for 1 hour. To this mixture 500µl of chloroform : isoamyl alcohol mixture was added and mixed well for 15 min. Then centrifuged at 12,000 rpm for 15 minutes and aqueous phase was transferred to another eppendorf tube without disturbing the inter phase. Twice the volume of ice-cold absolute alcohol and 1/10th volume of sodium acetate were added and kept for overnight incubation.

The mixture was centrifuged (12,000 rpm for 5 minutes) and the supernatant was discarded. The pellet was washed in 500µl of 70 per cent ethanol. Alcohol was discarded and DNA was air-dried completely. Then the DNA pellet was dissolved in 120-500µl of TE buffer (Appendix II) and stored at -20°C (Samsung RS21HUTPN1 -20 Freezer).

3.2.2.2 CTAB method of Genomic DNA Isolation from weedy rice seed

0.5 grams of weedy rice panicles were chilled and ground into a fine powder in liquid nitrogen using a sterile pre-chilled mortar and pestle. The powdered sample was transferred to eppendorf tubes. Added 150µl of extraction buffer (Appendix III) and 20µl of α-amylase (15mg/mL) then kept at 65°C for 30 min. in waterbath (RWB-01, ROTEK, India). Later 20µl of chloroform is added and mixed vigourously for 30 sec. Then centrifuged the tubes at 1660 g for 1 sec. at room temperature (Hermle Centrifuge Z 326 K, Germany) and added 20µl of 5 mol/L pottassium acetate into the mixture and mixed for 10 sec. Again centrifuged the tubes at 1660 g for 10 min. at room temperature. After centrifugation, 100µl of supernatant was carefully transferred into another eppendorf tube. 80µl of ice cold isopropanol was added to the supernatant and mixed by inversion several times. Centrifugation was done at 3000 g for 15 min. at room temperature. After centrifugation the supernatant

was discarded and washed the pellets with 70 per cent ethanol (v/v). Then dried the pellet at room temperature and dissolved in 50µl TE buffer and stored at -20°C (Samsung RS21HUTPN1 -20 Freezer).

3.2.2.3 Qualitative analysis of DNA samples

Agarose gel electrophoresis was done for the qualitative analysis of isolated DNA samples. Horizontal gel electrophoresis unit (Wide Mini-Sub Cell GT Cell, BIORAD, USA) was used to run the samples on the gel to determine the integrity and quality of genomic DNA. Aliquot of DNA sample in the following procedure were loaded into separate wells.

1µl 1kb ladder (Himedia) and 10 µl sample + 2 µl 6x Loading Buffer

It was loaded on 0.8 per cent agarose gel (melting 0.8 g of agarose in 100 ml of 1X TAE buffer (Appendix IV). The gel was run at 70 V until the dyes migrated 3/4th of the distance through the gel. The gel was visualized using the gel documentation system (Gel Doc™ XR+ Gel Documentation System, BIORAD, USA) using 'Image Lab Software'.

3.2.2.4 Quantification of DNA

The absorbance of the isolated DNA sample was recorded to determine the quantity and quality of DNA. UV- Visible Double Beam Spectrophotometer (AV - 2701 SYSTRONICS, INDIA) was used to measure the absorbance of the sample. Spectrophotometer was first calibrated to blank (The Optical Density (OD) value is zero) at 260nm and 280nm wavelength with 2 ml TE buffer. The OD of 2µl DNA sample dissolved in 2 ml of TE buffer at respective wavelengths were recorded.

Since an absorbance value of 1.0 at 260 nm indicates the presence of 50 ng μl^{-1} of double stranded DNA, the concentration of DNA in the extracted sample was estimated by using the following formula:

$$\text{Amount of DNA } (ng\mu l^{-1}) = (A_{260} \times 50 \times \text{dilution factor}) / 1000$$

(Where A_{260} is absorbance reading at 260 nm)

DNA quality was determined by the ratio taken between A_{260}/ A_{280} readings. (If the ratio is 2 or above 2, it indicates high RNA contamination in the sample and if the ratio is less than 1.8, protein contamination or phenol in the sample).

3.2.3 PCR Amplification of Genomic DNA with Degenerate Primers

The genomic DNA of various phases and parts of weedy rice biotypes were amplified using the designed degenerate primers for the shattering genes by two succeeding reactions. Primers used in the first PCR reaction were *sh4* and *qsh1*. The components of the mixture were optimized as listed below:

Water	:	13 µl
10x Reaction buffer (Hibuffer) : (Tris with 15 mM $MgCl_2$)		2µl
dNTPs (2.5 mM each)	:	1µl
Forward primer (10 µM)	:	1µl
Reverse primer (10 µM)	:	1µl
Template DNA (50 ngμl^{-1})	:	1µl
Taq Polymerase (1Uμl^{-1})	:	1µl
Total volume	**:**	**20µl**

PCR was carried out in PCR machine (T100™ Thermal Cycler, BIORAD, USA). PCR programme was set with initial denaturation at 94°C for 4 min. followed by 40 cycles each of denaturation at 94°C for 30 sec., appropriate annealing for 40 sec. and extension at 72°C for 45 sec. Final extension was done at 72°C for 5 min. Only the annealing temperature varied with primers. The various primers and their annealing temperature are listed out in Table 4. The amplified products along with 100bp ladder from 'Himedia' were separated on agarose gel (1.5 per cent). The gel was viewed under gel documentation system (Gel Doc™ XR+ Gel Documentation System, BIORAD, USA, BIORAD, USA).

3.2.4 RNA Isolation

RNA isolation was done for both weedy rice and cultivated rice variety *Uma* from leaf and culm at seedling, tillering, and flowering stages. Both CTAB and TRIZOL method were used for extraction of high quality total RNA, and checked

for its quality. All the materials used for RNA extraction were treated in 3 per cent hydrogen peroxide overnight and autoclaved twice for sterilization. The double distilled water was also autoclaved twice for making it RNase free.

3.2.4.1 CTAB Method for RNA isolation

β- mercaptoethanol (100-200µl) was added fresh to 5 ml CTAB extraction buffer and briefly vortexed. The solution was pre-heated to 65°C in waterbath (RWB-01, ROTEK, India). The samples were frozen and ground into a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. 500 mg of the powdered samples were immediately transferred to Falcon tube containing 5 ml of pre-warmed CTAB extraction buffer (Appendix V). The content was vortexed using a vortex shaker (SPINIX MC-01, Tarsons, India) and then incubated the mixture for 15 min. at room temperature by intermittent shaking for every 5 min. To the homogenate, an equal volume of chloroform:isoamyl alcohol (24:1) was added and vortexed several times for 1 min. The homogenate was centrifuged (Hermle Z 326 K centrifuge, Germany) at 12,000g force for 15 min. at 4°C. The upper phase was transferred to a fresh Falcon tube and 1/3 volume of 8M LiCl was added. The mixture was then incubated at 4°C overnight for the precipitation of RNA. After incubation, the precipitated RNA was centrifuged at 12,000 g force for 20 min. at 4°C for obtaining the pellet. The supernatant was decanted by using a micropipette. The pellet was then resuspended in 50µl RNase free water. The extracted RNA samples were stored at -80°C (Panasonic MDF U55V-PE -80 Freezer, India).

3.2.4.2 TRIZOL Method for RNA isolation

The samples were frozen and ground into a fine powder in liquid nitrogen using a pre-chilled mortar and pestle and collected in eppendorf tubes. Immediately added 1ml of TRIZOL to the homogenized tissue then vortexed using vortex shaker (SPINIX MC-01, Tarsons, India) and incubated at room temperature for 10 min. Mixture was centrifuged (Hermle Z 326 K centrifuge, Germany) at 13,000 rpm for 10 min. at 4°C to remove extracellular material. After centrifugation, the supernatant was transfered to new eppendorf tubes. 0.2ml of chloroform per 1ml TRIZOL

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was added. Vigorous shaking was done by hand for 15 seconds. The mixture was incubated at room temperature for 3 min. Samples were centrifuged 13000 rpm for 15min. at 4°C for phase separation. The upper aqueous phase was transfered to new tubes (50-60 per cent of TRIZOL vol.). RNA is precipitated by mixing with 0.5 ml isopropanol per 1ml TRIZOL and incubated at room temperature for 10 min. Centrifugation was done at 13,000 rpm for 10 min. at 4°C to obtain pellets. Supernatant was removed and the pellets were washed with 1ml 75 per cent EtOH (diluted with DEPC treated water). Vortexed once and centrifuged at 7500 x g for 5 min. at 4°C. Supernatant was discarded and dried the pellets for 5 min. at room temperature. The pellet was dissolved in 0.05 ml DEPC treated water and incubated at 55°C for 10 minutes. The extracted RNA samples were stored at -80°C (Panasonic MDF U55V-PE -80 Freezer, India).

The integrity of the total RNA was determined by running 10µl aliquot of RNA in agarose gel (1.5 per cent) as described in section 3.2.2.3.

The absorbance reading of the extracted RNA was determined using spectrophotometer as described in section 3.2.2.4. Since an absorbance value of 1.0 at 260 nm indicates the presence of 40 ngμl^{-1} of RNA, the concentration of RNA present in an aliquot was estimated by using the following formula:

$$\text{Amount of RNA (ng}\mu l^{-1}) = A_{260} \times 40 \times \text{dilution factor}$$

(Where A_{260} is absorbance reading at 260 nm)

RNA quality was determined by the ratio taken between A_{260}/ A_{280} readings. (The ratio for pure RNA $A_{260/280}$ is ~2 and if the ratio is less than 1.8, it indicates protein contamination or phenol in the sample).

3.2.5 Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

RT-PCR is the most sensitive technique for the detection and quantification of mRNA, compared to other available methods such as Northern blot analysis and RNase protection assay. It can be used for quantification of mRNA levels from lesser amount of samples.

RT-PCR was carried out using First strand cDNA synthesis kit K1612 (Thermo Scientific™). RT-PCR is essentially a two-step process. The first step involves the synthesis of cDNA from single- stranded mRNA using reverse transcriptase enzyme and the second step is the amplification of cDNA by PCR. A sterile working environment was maintained to avoid contamination so that all the materials used were pre-treated with 3 per cent hydrogen peroxide overnight and autoclaved twice.

3.2.5.1 Synthesis of cDNA Using Thermo Scientific First Strand cDNA Synthesis Kit

After thawing, mixed and briefly centrifuged the components of the kit and added the following reagents into a sterile, nuclease-free PCR tube on ice in the indicated order:

Template RNA	:	2µl
Oligo $(dT)_{18}$ primer	:	1µl
Water (nuclease-free)	:	8µl

The tubes were gently mixed, centrifuged briefly and incubated at 65°C for 5 min. to denature RNA secondary structures and allow more efficient priming and cDNA synthesis. After incubation, the samples were chilled on ice, span down and placed the vial back on ice and added the following components in the indicated order:

5X Reaction Buffer	:	4µl
RiboLock RNase Inhibitor (20 U/L)	:	1µl
10 mM dNTP Mix	:	2µl
M-MuLV Reverse Transcriptase (20 U/L)	:	2µl
Total volume	**:**	**20µl**

The contents were gently mixed and centrifuged briefly after adding the components. Incubated for 60 min. at 37°C and terminated the reaction by heating at 70°C for 5 min. and stored at -20°C (Samsung RS21HUTPN1 -20 Freezer), until PCR amplification.

3.2.5.2 PCR Amplification of cDNA with Degenerate Primers

The cDNA samples were subjected to PCR using the designed degenerate primers for isolating the genes by two succeeding reactions already mentioned in section 3.2.3., except that 2µl of cDNA was used as template instead of genomic DNA and the total volume was 20µl. The components of the mixture for each reaction were optimized are listed here:

Water	:	12 µl
10x Reaction buffer (Hibuffer) : (Tris with 15 mM $MgCl_2$)		2µl
dNTPs (2.5 mM each)	:	1µl
Forward primer (10 µM)	:	1µl
Reverse primer (10 µM)	:	1µl
Template DNA (50 ngμl^{-1})	:	2µl
Taq Polymerase (1Uμl^{-1})	:	1µl
Total volume	**:**	**20µl**

The PCR programme was set with initial denaturation temperature of 94°C for 4 min., followed by 35 cycles of denaturation at 94°C for 30 sec. appropriate annealing for 40 sec. and extension at 72°C for 45 sec. Final extension was set at 72°C for 5 min. Only the annealing temperature varied with primers. The various primers and their annealing temperature are listed out in Table 4. The PCR products were separated on agarose gel (1.5 per cent) and the gel was observed using gel documentation system. (Gel Doc™ XR+ Gel Documentation System, BIORAD, USA, BIORAD, USA).

3.2.6 Sequencing of the Amplicons Produced after PCR

The amplicons produced by the degenerate primer pair was eluted from the agarose gel and purified using HipurA™ PCR product and gel purification combo kit (Himedia). The eluted product after cloning was given to SciGenom Labs Pvt. Ltd., Kochin for sequencing.

3.2.6.1 Gel Elution and Purification Using HipurA™ PCR Product and Gel purification Combo Kit (Himedia)

After PCR, the gel piece containing the amplicon was taken, weighed and placed in a centrifuge tube. According to the weight of the gel slice, added 3 volumes of Combo binding buffer per gel slice volume. The mixture was then incubated at 55-60°C for 7 min. by mixing the contents of the tube after every 2-3 min., until the gel has melted completely. The gel-combo binding buffer mixture obtained after the incubation was loaded (700µl of the sample mixture was loaded at a time) on to a HiElute Miniprep spin column (capped) and then centrifuged at 12,000 rpm (10,000 X g) for 1 min. at room temperature. The flow-through was discarded and placed the column back into the same collection tube. 300µl of the Combo binding buffer was then added into the column and centrifuged at 12,000 rpm (10,000 X g) for 1 min. at room temperature. Discarded the flow-through and reused the collection tube. 700µl of the diluted wash solution (which was diluted with ethanol in 1:4 ratio) was added and centrifuged at 12,000 rpm for 1 min. at room temperature. After discarding the flow-through, reused the collection tube and centrifuged the empty column at maximum speed (≥ 14,000 rpm) for 2 min., to dry the column membrane, for the complete removal of ethanol. The column was then placed into a new uncapped collection tube and 30-50µl of Elution buffer (ET- 10 mM Tris-Cl, pH= 8.5) was added according to the desired concentration of the trial product, directly into the column membrane. After an incubation for 1 min. at room temperature, the column was centrifuged at maximum speed (≥ 14,000 rpm) for 1 min., for the elution of the PCR product (DNA). The eluate was then stored at -20°C.

3.2.6.2 Cloning of PCR products for Sequencing

The most efficient method for getting good sequences of the PCR products is cloning. It is essentially a four-step process including competent cell preparation, ligation of the PCR products, transformation of the ligated products into competent cells (*E. coli* DH5α) and Plasmid isolation from transformed colonies.

3.2.6.2.1 Competent Cell Preparation

E. coli cells treated with cold calcium chloride ($CaCl_2$) takes up plasmid DNA by transformation. $CaCl_2$ is thought to enhance binding of plasmid DNA to cell surface by affecting plasma membrane permeability.

The *E. coli* strain used for the transformation was *E. coli* DH5α. From a freshly streaked LB agar (2 per cent) plate, *E. coli* was inoculated into 50 ml LB broth directly. The culture was then incubated at 37°C overnight with shaking. After incubation, 500µl of the culture was inoculated into 50ml LB broth in 250mL flask and allowed to grow for 2-3 hours till the OD - 600 reached 0.3 to 0.4 (silky appearance). The cells were then harvested by spinning the bacterial culture taken in 2ml Eppendorf tubes at 4,000 rpm for 5 min. The supernatant was discarded and the pelleting was also repeated using fresh culture until sufficient amount of bacterial pellet was obtained. The pellet was then resuspended in 1 ml chilled 0.1 M $CaCl_2$ and centrifuged at 4,000 rpm for 5 min. 4°C. The pellet was again resuspended in 1 ml chilled 0.1 M $CaCl_2$ and kept on ice for 30 min. After centrifuging at 4,000 rpm for 5 min. at 4°C, resuspended the pellet in 200µl chilled 0.1M $CaCl_2$ and stored at 4°C.

3.2.6.2.2 Ligation of PCR products

The ligation of the PCR products was done using TA Cloning® Kit (Invitrogen). One vial containing pCR®2.1 vector was centrifuged for collecting all the liquid at the bottom of the vial. The components of the ligation mixture are listed below:

Fresh PCR product	:	2µl
5X T4 DNA Ligase Reaction Buffer	:	2µl
pCR®2.1 vector (25 ng μl^{-1})	:	2µl
Water	:	3µl
ExpressLink™ T4 DNA Ligase (5U)	:	1µl
Total volume	**:**	**10µl**

The ligation mixture was then incubated for 20 min. at room temperature and stored at -20 °C.

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3.2.6.2.3 Transformation of Ligated PCR products into Competent cells

The tubes containing ligation reactions were spun briefly and kept on ice. 10µl of the ligated products were added to the tubes containing 200µl of competent *E. coli* DH5*α* cells and inverted the tubes gently to mix and placed them on ice for 30 min. The cells were then allowed to heat shock at 42°C for 2 min. in waterbath without shaking and immediately kept on ice for 2 min. To each of the tubes containing the cells, 800 µl of LB broth was added. The culture was then incubated for 1 h at 30°C with shaking at 50 rpm in shaker cum incubator (ROTEK-LES, India). After the incubation, 200 µl of the transformed culture was poured on LB/Amp/IPTG/X-Gal plates and spread plating was done using a sterile L- rod. The culture plates were then allowed to dry and incubated at 30°C overnight in shaker cum incubator.

3.2.6.2.4 Plasmid isolation from transformed colonies

Transformed colonies from freshly spread plates were used to inoculate 5 ml LB broth (containing appropriate antibiotics) taken in test tubes. The culture was incubated at 37°C overnight with shaking at 180 rpm in shaker cum incubator. After incubation, the culture was poured into 1.5 ml tube and centrifuged at 4000 rpm for 4 min. to pellet out the cells. The supernatant was removed and resuspended the bacterial pellets in 200µl GET buffer (Appendix VI). 300µl freshly prepared Lysis buffer (Appendix VII) was added and mixed the contents of the tubes by gentle inversion. After an incubation on ice for 5 min., the solution was neutralized by adding 300µl Neutralizing solution (Appendix VIII) and mixed by gentle inversion, followed by incubation on ice for 5 min. The solution was then centrifuged at 15,000 rpm for 10 min. at room temperature, for the removal of cellular debris and transferred the supernatant to a clean tube. To the supernatant, 400µl of Chloroform was added and mixed by inversion for 30s. The solution was then centrifuged at 13,000 rpm for 1 min. to separate phases and transferred the upper aqueous phase to a fresh tube. This step was done 2 times, and an equal volume of 100 per cent isopropanol was added. After mixing the contents by inversion, it was centrifuged at maximum speed (14,000 rpm) for 10 min. at room temperature and removed the isopropanol. The pellet was then washed with 500µl 70 per cent ethanol and centrifuged at 10,000 rpm for 5 min. After the centrifugation, the pellet was allowed to air dry for 30 min. in laminar hood. The pellet was resuspended in 60µl double distilled water and stored at -80°C for further use.

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RESULTS

4. RESULTS

The main objective of the study was to identify the genes, related to shattering in weedy rice biotypes of Kerala *viz. sh4* and *qsh1*. The gel profile of PCR products from DNA/RNA were observed and PCR products were sequenced. Evolutionary study was conducted through phylogenetic tree analysis and the candidate genes were examined for their expression profiles by doing RT-PCR using the degenerate primers. The results related to the study are elaborated in this chapter.

4.1 IDENTIFICATION OF SHATTERING GENE

4.1.1 Designing of Degenerate Primers

Degenerate primers for the genes reported to regulate shattering *viz., sh4* and *qSh1* was designed based on the conserved regions in the multiple aligned nucleotide sequences using Clustal X and Sequence Manipulation Suite. The details of the designed primers are given in Table 1.

4.1.1.1 Primer Analysis

The analysis of designed primers was done by using Oligo Calc programme. The primers selected provided the desirable GC content ranging from 40 - 60 per cent except *sh4* FW1, *sh4* FW3, *sh4* RW3, *qsh1* FW4 and *qsh1* RW4. All the primers lineup the optimal length of the primers ranging from 18-22bp. The annealing temperatures of the primers ranging from 50 - 81, and none of the designed primers exhibited any hairpin formation and 3' complementarity.

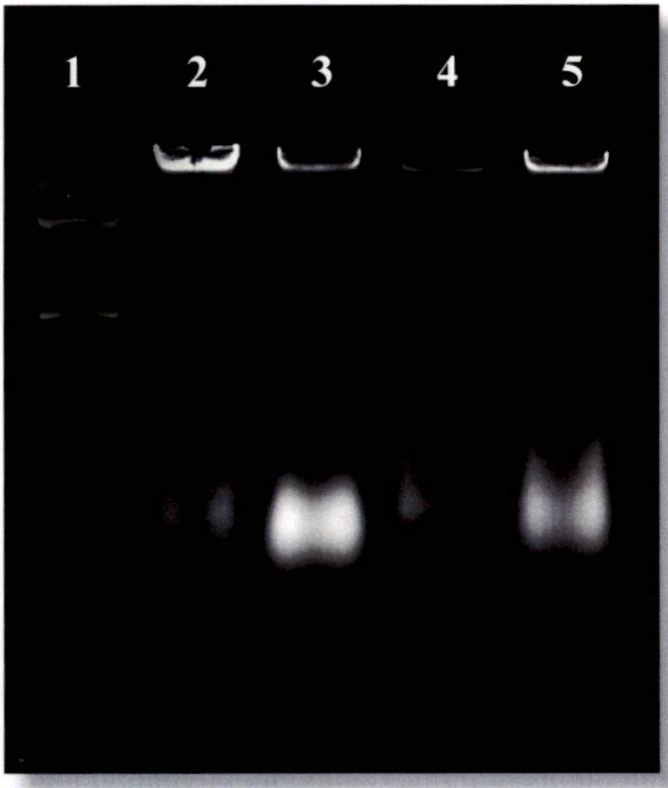
4.1.2 DNA Isolation

Genomic DNA of both flag leaf and panicles of weedy rice and *Uma* were extracted. The agarose gel electrophoresis (0.8%) of the extracted genomic DNA showed the presence of non-sheared bands having good quality (Plate 5).

Table 1. Degenerate primers designed for *sh4* and *qsh1*

Target Gene	Primer Name	Sequence	Tm°C	GC%	nt	Extinction coefficient (l mol^{-1} cm^{-1})	Molecular weight (g/mol)	nmol	µg/ OD260	Ruler (bp)
sh4	FORWARD									
	FW1	GACRAAATATCAAACGTRTGAT	56.9	31.8	22	231600	6775.5	4.3	29.3	17-38
	FW2	GATTGAGTATATGCGAGRCAT	57.6	40.5	21	216350	6517.3	4.6	30.1	71-95
	FW3	CCTCTMAAMAATTTAACACT	50.8	30	20	191000	6004	5.2	31.4	106-136
	FW4	GCAGTGCAGTTACAAACCG	61.3	52.6	19	187800	5821.9	5.3	31	598-620
	REVERSE									
	RW1	CGAGCGTTTAGCTGGTTAAGTA	61.6	45.5	22	217400	6805.5	4.6	31.3	803-824
	RW2	GACAGACCTGACACTGCTCAT	61.3	52.4	21	199000	6375.2	5	32	700-720
	RW3	GCGCGTCGGCGTCGCCCGTA	81.6	80	20	179200	6111	5.6	34.1	782-802
	RW4	GSGAGCAGAGGCGTTCCAT	68.6	63.2	19	185350	5873.9	5.4	31.7	778-796
qsh1	FORWARD									
	FW1	GCTCGGAAAGTGCGGAGCCT	71.1	65	20	190900	6183.1	5.2	32.4	13-33
	FW2	CGAGGAGAAGAGACCCAC	59.2	61.1	18	186800	5551.7	5.4	29.7	130-154
	FW3	GCTCCACCACCATCCGCA	70.2	66.7	18	160100	5349.5	6.2	33.4	264-281
	FW4	GCTCACTGCGCTGCGCCTCT	73.7	70	20	164600	6020.9	6.1	36.6	68-87
	REVERSE									
	RW1	GCGCCGCCGACGTCGCAGAT	79.5	75	20	183300	6104	5.5	33.3	769-788
	RW2	CGTGATCAACGACGTCAT	60	50	18	174900	5483.6	5.7	31.4	860-877
	RW3	CGACGCCGTCCATCGGAT	71	66.7	18	165700	5460.6	6	33	839-856
	RW4	CGGCCGAGCACGGCGGCGTA	81.5	80	20	188400	6169	5.3	32.7	709-728

Degenerate code bases : **M** - AC **S** - CG **V** - ACG **B** - CGT
R - AG **Y** - CT **H** - ACT **N** - ACGT
W - AT **K** - GT **D** - AGT



1 - 1kb Ladder
2 - Flag leaf of Weedy rice
3 - Grain of Weedy rice
4 - Flag leaf of *Uma*
5 - Grain of *Uma*

Plate 5. Agarose gel profiling of genomic DNA from leaf and panicles of weedy rice and *Uma* variety

Table 2. Quality and quantity of isolated genomic DNA

Sl. No.	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	DNA Yield ($ngµl^{-1}$)
1	Flag leaf of Weedy Rice	0.099	0.054	1.83	2970
2	Panicles of Weedy rice	0.067	0.038	1.76	2010
3	Flag leaf of *Uma* Variety	0.102	0.049	1.78	2610
4	Panicles of *Uma* Variety	0.096	0.054	1.79	2910

Through spectrophotometric method, the absorbance reading of the extracted genomic DNA showed good quality as well as quantity (Table 2).

4.1.3 PCR Analysis of Genomic DNA with Degenerate Primers

Genomic DNA of flag leaf from both weedy rice and *Uma* were used as template. Two of the *sh4* primers and one of the *qsh1* primers designed for shattering yielded amplicons of expected size *viz.* 649bp, 696bp and 747bp respectively. (Plate 6). The result confirmed that primers are functional and both *sh4* and *qsh1* genes are present in weedy rice and variety *Uma*.

4.1.5 RNA Isolation

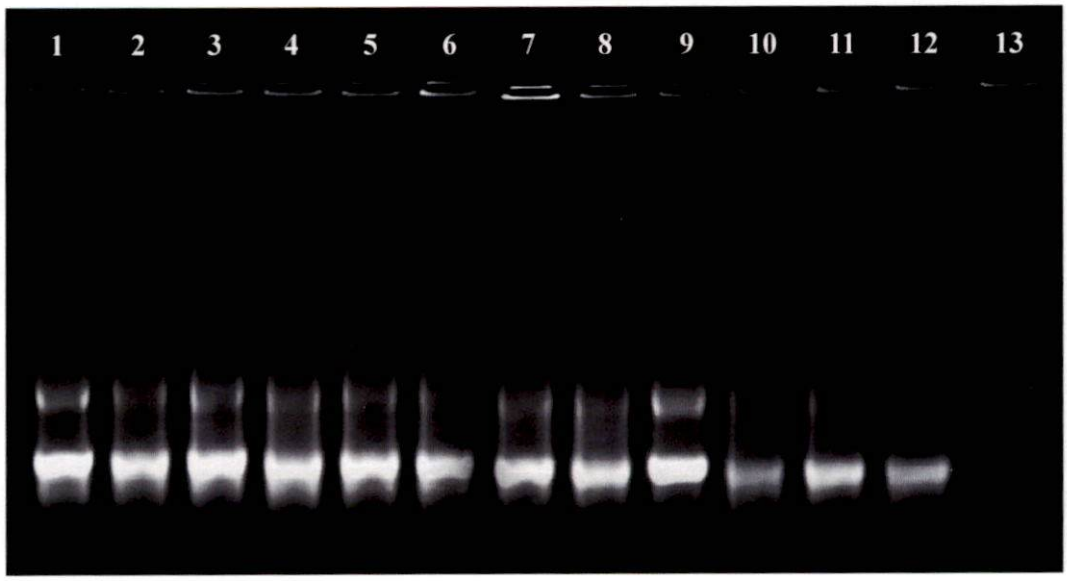
The total RNA was extracted from both weedy rice and cultivated rice variety *Uma* from leaf and culm at seedling, tillering, and flowering stages. Distinct intact rRNA (ribosomal RNA) bands with no RNA degradation with lesser amount of genomic DNA contamination was observed on agarose gel (1.5 %) showing good quality RNA extraction using CTAB method. (Plate 7). All the RNA samples were used to prepare cDNA and for PCR.

For expression studies, Weedy rice flag leaf, stem, root, and grain tissues were used for extraction of total RNA. Distinct intact rRNA bands with no RNA degradation was observed with lesser amount of DNA contamination on agarose

1 - Flag leaf of weedy rice *sh4* FW1 x RW1
2 - Flag leaf of weedy rice *sh4* FW2 x RW2
3 - Flag leaf of weedy rice *sh4* FW3 x RW3
4 - Flag leaf of weedy rice *sh4* FW4 x RW4
5 - Flag leaf of weedy rice *qsh1* FW1 x RW1
6 - Flag leaf of weedy rice *qsh1* FW2 x RW2
7 - Flag leaf of weedy rice *qsh1* FW3 x RW3
8 - Flag leaf of weedy rice *qsh1* FW4 x RW4
9 - 100bp DNA Ladder
10 - Flag leaf of *Uma sh4* FW1 x RW1
11 - Flag leaf of *Uma sh4* FW2 x RW2
12 - Flag leaf of *Uma sh4* FW3 x RW3
13 - Flag leaf of *Uma sh4* FW4 x RW4
14 - Flag leaf of *Uma qsh1* FW1 x RW1
15 - Flag leaf of *Uma qsh1* FW2 x RW2
16 - Flag leaf of *Uma qsh1* FW3 x RW3
17 - Flag leaf of *Uma qsh1* FW4 x RW4
18 - Actin Control

19 - Panicles of Weedy rice *sh4* FW1 x RW1
20 - Panicles of Weedy rice *sh4* FW2 x RW2
21 - Panicles of Weedy rice *sh4* FW3 x RW3
22 - Panicles of Weedy rice *sh4* FW4 x RW4
23 - Panicles of Weedy rice *qsh1* FW1 x RW1
24 - Panicles of Weedy rice *qsh1* FW2 x RW2
25 - Panicles of Weedy rice *qsh1* FW3 x RW3
26 - Panicles of Weedy rice *qsh1* FW4 x RW4
27 - 100bp DNA Ladder
28 - Panicles of *Uma sh4* FW1 x RW1
29 - Panicles of *Uma sh4* FW2 x RW2
30 - Panicles of *Uma sh4* FW3 x RW3
31 - Panicles of *Uma sh4* FW4 x RW4
32 - Panicles of *Uma qsh1* FW1 x RW1
33 - Panicles of *Uma qsh1* FW2 x RW2
34 - Panicles of *Uma qsh1* FW3 x RW3
35 - Panicles of *Uma qsh1* FW4 x RW4
36 - Actin Control

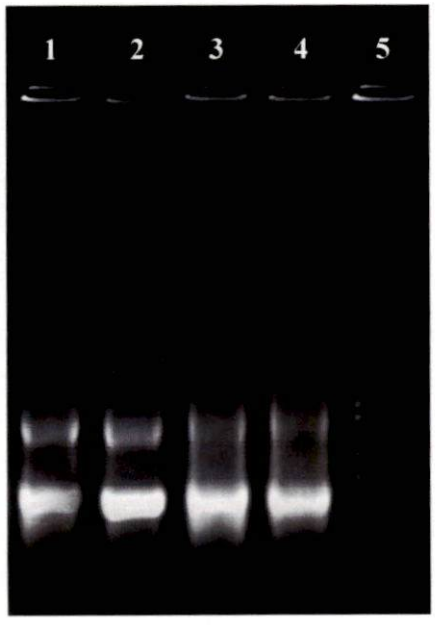
Plate 6. Agarose gel profiling of PCR product from genomic DNA of leaf and panicles of weedy rice and *Uma* variety



Legends

1 - Leaf at seedling stage of weedy rice
2 - Leaf at tillering stage of weedy rice
3 - Flag leaf of weedy rice
4 - Culm at seedling stage of weedy rice
5 - Culm at tillering stage of weedy rice
6 - Culm at flowering stage of weedy rice
7 - Leaf at seedling stage of *Uma*
8 - Leaf at tillering stage of *Uma*
9 - Flag leaf of *Uma*
10 - Culm at seedling stage of *Uma*
11 - Culm at tillering stage of *Uma*
12 - Culm at flowering stage of *Uma*
13 - 100bp DNA Ladder

Plate 7. Agarose gel profiling of total RNA from different tissues of various stages in weedy rice and *Uma* variety



Legends

1 - Flag leaf of weedy rice
2 - Culm of weedy rice
3 - Root of weedy rice
4 - Grain of weedy rice
5 - 100bp ladder

Plate 8. Agarose gel profiling of total RNA from different tissues of weedy rice for expression studies

Table 3. Quality and quantity of isolated total RNA

Sl. No.	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	RNA Yield ($ng\mu l^{-1}$)
1	Leaf at seedling stage of weedy rice	0.198	0.095	2.08	4752
2	Leaf at tillering stage of weedy rice	0.184	0.091	2.02	4416
3	Flag leaf of weedy rice	0.122	0.061	2.00	2928
4	Culm at seedling stage of weedy rice	0.138	0.072	1.92	3312
5	Culm at tillering stage of weedy rice	0.101	0.052	1.94	2424
6	Culm at flowering stage of weedy rice	0.115	0.059	1.95	2760
7	Leaf at seedling stage of *Uma* variety	0.106	0.053	2.00	2544
8	Leaf at tillering stage of *Uma* variety	0.115	0.054	2.13	2760
9	Flag Leaf of *Uma* variety	0.107	0.051	2.10	2568
10	Culm at seedling stage of *Uma* variety	0.139	0.071	1.96	3336
11	Culm at tillering stage of *Uma* variety	0.119	0.062	1.92	2856
12	Culm at flowering stage of *Uma* variety	0.099	0.054	1.83	2376
13	Culm of weedy rice	0.107	0.059	1.81	2568
14	Root of weedy rice	0.101	0.057	1.77	2424
15	Grain of weedy rice	0.112	0.062	1.81	2688

gel (1.5%) showing good quality RNA extraction (Plate 8). Further absorbance reading of the extracted RNA by using spectrophotometric method revealed good quality and quantity of RNA (Table 3).

4.1.6 RT-PCR with Degenerate Primers

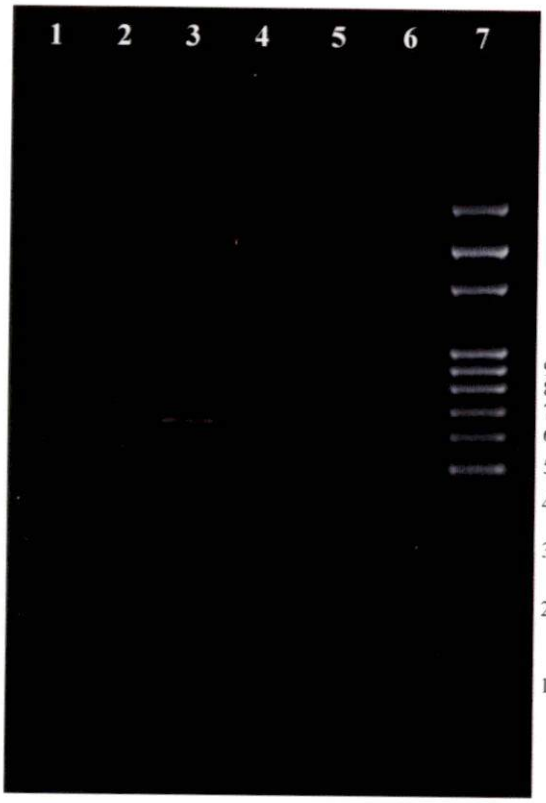
The reverse transcription of the extracted RNA from weedy rice and cultivated rice variety *Uma,* from leaf and culm at seedling, tillering, and flowering stages, were carried out for the synthesis of the first strand cDNA. PCR was done with the resulted primers in genomic DNA samples (*ie., sh4* FW2, RW2 and *qsh1* FW2, RW2 primers). Other RNA samples of weedy rice flag leaf, culm, root and grain for expression studies were also used for cDNA synthesis and further PCR.

When cDNA samples of weedy rice leaf and culm at seedling, tillering and flowering stages were used as template, the reaction did produce an amplicon of size ~690bp with the primer combination of *sh4* FW2 x RW2 in flag leaf. Also, no bands were seen in other stages of leaf at seedling and tillering and culms at seedling, tillering and flowering stages. (Plate 9).

When cDNA from cultivated variety *Uma* leaf and culm at seedling, tillering, and flowering stages were used as template, the reaction did not produce any amplicons with the primer combination of *sh4* FW2 x RW2 (Plate 10).

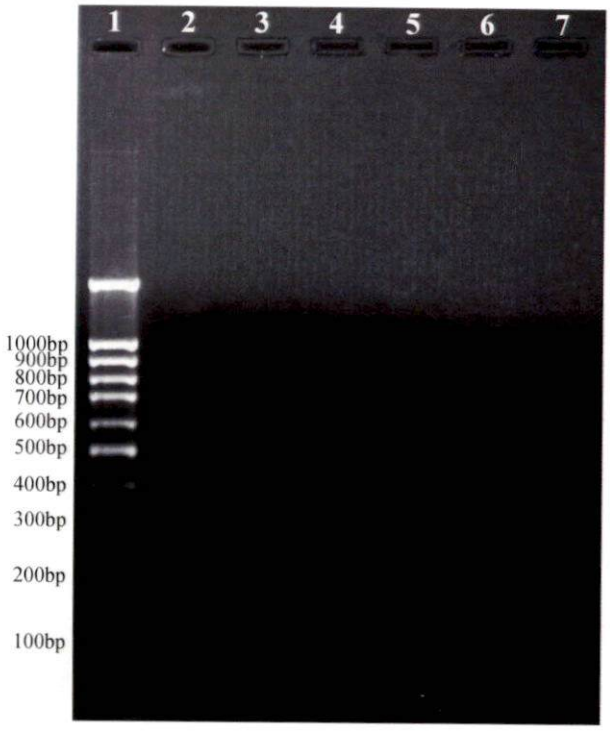
For the reaction with the primer combination of *qsh1* FW2 x RW2 in samples of weedy rice leaf and culm at seedling, tillering, and flowering stages as templates, the reaction did produce an amplicon of size ~750bp in flag leaf. But no bands were observed in other stages of leaf and culms. (Plate 11). Lane 3 shows the expected amplicon product.

Like in the case of *sh4* primers, cultivated variety *Uma* leaf and culm at seedling, tillering and flowering stages of cDNA were used as template, the reaction did not produce any amplicons with the primer combination of *qsh1* FW2 x RW2 (Plate 12).



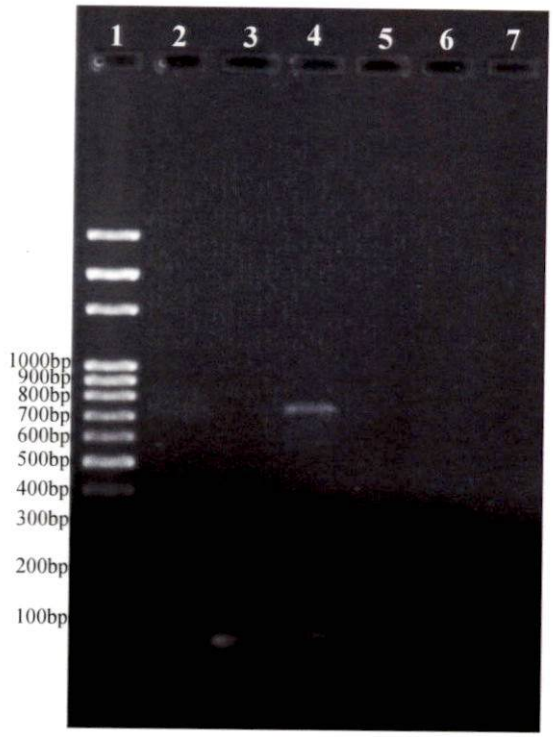
Legends
1 - Leaf at seedling stage of weedy rice
2 - Leaf at tillering stage of weedy rice
3 - Flag leaf of weedy rice
4 - Culm at seedling stage of weedy rice
5 - Culm at tillering stage of weedy rice
6 - Culm at flowering stage of weedy rice
7 - 100bp ladder

Plate 9. Agarose gel profiling of PCR product from cDNA of leaf and culm at seedling, tillering, and flowering stages of weedy rice using *sh4* FW2 x RW2 primers



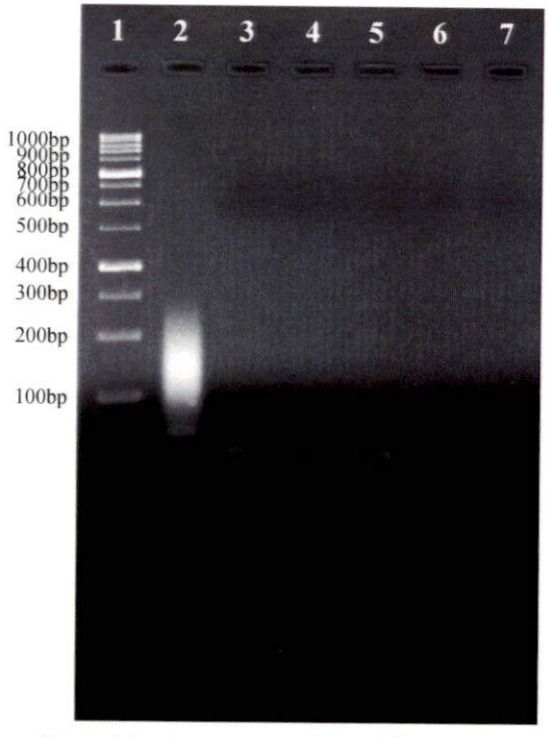
Legends
1 - 100bp ladder
2 - Leaf at seedling stage of *Uma*
2 - Leaf at tillering stage of *Uma*
3 - Flag leaf of *Uma*
4 - Culm at seedling stage of *Uma*
5 - Culm at tillering stage of *Uma*
6 - Culm at flowering stage of *Uma*

Plate 10. Agarose gel profiling of PCR product from cDNA of leaf and culm at seedling, tillering, and flowering stages of *Uma* variety using *sh4* FW2 x RW2 primers



Legends
1 - 100bp ladder
2 - Leaf at seedling stage of weedy rice
3 - Leaf at tillering stage of weedy rice
4 - Flag leaf of weedy rice
5 - Culm at seedling stage of weedy rice
6 - Culm at tillering stage of weedy rice
7 - Culm at flowering stage of weedy rice

Plate 11. Agarose gel profiling of PCR product from cDNA of leaf and culm at seedling, tillering, and flowering stages of weedy rice using *qsh1* FW2 x RW2 primers



Legends
1 - 100bp ladder
2 - Leaf at seedling stage of *Uma*
2 - Leaf at tillering stage of *Uma*
3 - Flag leaf of *Uma*
4 - Culm at seedling stage of *Uma*
5 - Culm at tillering stage of *Uma*
6 - Culm at flowering stage of *Uma*

Plate 12. Agarose gel profiling of PCR product from cDNA of leaf and culm at seedling, tillering, and flowering stages of *Uma* variety using *qsh1* FW2 x RW2 primers

Sequences of the amplicon after sequencing are given below.

sh4 FW - TGTGGGGGTTCAATTGTTGTTAAAAAGCTATTGGTAGCAGTAT
CTAGTATGGCGTGGCAGGCCGGTGCAGAAGGGGCCCCGGGT
TTTGCCCGGAATTTCGCGTAGGACGCCCCGGCAGCTAGCGGA
CGCTTTGCATTTGCATGCATGGTGCTCTCTACTTCTCCCTCCG
TTCCCACGGCAAATTGCAAAAACTCGTAACGTATTTGGCACC
ATTAGCCTAAGGTAGTAGTATCAGCGTGTTACAGAAAGAAAA
TAGTTAAGTAGCAGTAATTAATTAAAGCCTGTGATTAAGGTAA
AGCAGCAGTGCAGTTACAAACCGAAACAAACAAGAGTCAGC
GAACTGAAAAGAAGACCGGGAAGAAGAAGAAGAAGGCGCG
CTTAGACCCGAACAGAGTTTTGATGAGCAGTGTCAGGTCTGT
CACATGAGAAGAGAGCGAAGTAGCTCAGCTCAGCCCCCACT
CGCTCACATGGAACGCCTCTGCTCGCCTCGACTACTTAACCA
GCTAAACGCTCGGTTGATTAGGAGAGGAAAAAAAACGAGGG
AAAAAACGGGTGGAAACACACGCAAACCACAACGCCGTGC
GGCCTTGTAAATACGGCCGTCCAATGACGCGCATCGCTCCCC
GAACACCAAACGCCTCAGCTTGCCTTGGCTCTCGCCAGTCGC
T (677bp)

sh4 RW - ACACGTCTTTTAATAAGACGAAATATCAAACGTATGATAAAAA
AAATCAATAACGCCATCTATTAAAAGACAGATTGAGTATATGC
GAGACATTTAATTATTTGCCACCTCTCAACAATTTAACACTCG
TGTGACATAGGAGTAGACGCATAGGGTAACAAATTGCTAAAC
GTCAAGTAATCTATGAGTAGCAACTAGCAAATACTTAAATATC
TCCTAATATGTTAGCATTGCACCAAAGTCTATAACTATATTGAG
CTTTGTCCTGTCCATGGTAAAGTGTGGGGTTAAAGTGTTAAA
AAGCTATTGGTAGCAGTATCTAGTATGGCGTGGCAGGCCGGT
GCAGATAAAAGGGGCCCCGGGTTTTGCCCGGAAATGAGGAG
AGGACGCCCCGGCAGCTAGCGGACGCTTTGCATTTGCATGCA
TGGTGCTCTCTACTTCTCCCTCCGTTCCCACGGCAAATTGCAA
AACGCATGCTAGTATTTGGCACCATTAGCCTAAGGTAGTAGTA
TCAGCGTGTTACAGAAAGAAAATAGTTAAGTAGCAGTAATTA
ATTAAAGCCTGTGATTAAGGTAAAGCAGCAGTGCAGTTACAA
ACCGAAACAAACAAGAGTCAGCGAACTGAAAAGAAGACCG
GGAAGAAGAAGAAGAAGAAAAAAAAAAGACCCGA (668bp)

qsh1 FW - TTTATAAGCAGTCAGCTGGGAAAAGCGAAGCGAGCCGGAAA
AAAGTTCCTCGAGTGTTGGTGCGTGCGCGTTATGTCGTCTGC
GGCTGGGGGGGGGGGTACGGCGTCGCCGAGTGGAGGGGCG
CGCGCGCCGAGGAGAAGACGGTACGCCGGTCATCTCTTGTTC
TTCACATCCTCGACACTTGGCCGGGGTGTGTTTTTCGCCATTG
TTGCGGCGTGGTGTAATTGATAATTGGGTCCCAGTTAGGGGG
ACAAGATTTCTTTTTTCAGGAAGTGGGGATTCCACCCCTATTT
TTTGTTCTTTGGTTGCCCCGAAACAAAAAGAGGGTTTGGGTT
CGCGGGGGTTTAATAGGAATGCTTTGTTTTTATACCGCAGAAG
ATTAATCTAGGATGGGTATTTGTGGCCATGGTTGCGCCTCTTT
CGGTTAACTAGGAGATTATCTTGTTTGTTTTTTCGGGTGGTAA
ACTTCACACGGAGGGTATCGGGGGGATGCTCCGGTTTGGGGA
AAAGGGCTTTTGTTCTTTACGAATCGGGGGGGTTGGGGGTCT
TTTTTTTCGGCGGGTAAAAATTTGTTCAAGGAAATCATGACCC
CCGCCGAAAAAAATG (606bp)

qsh1 RW - TGCTCCTTCTCTGTGTGATCTGTACGCGTAGCGGATCAGCCGG
AGCCGTAGCCGATGCAAGGAGCGAACTCGCCGGTGTCGTGC
GCGAGACGGCGGAGCTGGCTCCTCGGCTGGGCCACCCCGGC
GGGATCATTCGACCACGTTTCCTCGAGCCACAGCAGAAGCTT
CTGCGTTTTTTTCTGCTAGGCCCACAACAATAACTCATGCTCC
GTCGTATTTCTGCGAGTCCTGGGTTGTCTCGGTGTCATTTGAT
GACGTGAAAAACTCGAACTTCCTCCGTGTTTGTTATGACCCG
GGGGAAGGAGGTATGATCGGCGGGGGTGAGTTGTCGTTCAC
TGACAGAAACGCGAGGGCGCGGGTGTTCGCGCGAAAGGCAT
CGATCCCGTCCCCGCCGTTACGGCTTTTACTTTTTTTGACGAA
TGCATTCAGATTCGGACCGCCATCTCCGACACCGCGGCCACA
CTAGGTGATGGTATTGGAGAAAAATAGTACCCCGCCCCCCCC
CCCCGGGGGGGGGGGTTTTTTCCCGTCCTCCCTTCGGGTTGGG
GCGTGTTTGGCCCCCCCCCGCGGGGGGGAGAAAATAGGGTG
GCACCCCACTCTGGGGGTGGGTTTTTTTTCTTTCTGGGGGGG
GGGGGGTGGGGTGTATAAAAAAGATGGGGGGGGGGCAAAA
AAAAACACCCCAGAAAAAAAAAAAA (694bp)

4.1.7 Sequence Analysis of the Amplicons

The resultant sequence of the amplicon after sequencing was used for analysis using bioinformatic tools *viz.,* Sequence Manipulation Suite, tblastx, NCBI conserved Domain Search and phylogenetic tree construction using Clustal Phylogeny program.

The Sequence Manipulation Suite was used for combining and aligning the forward and reverse sequences. The BLAST programme used was tblastx i.e., translated nucleotide query is used to search in translated amino acid database. In case of cDNA from flag leaf of weedy rice, the results showed that the *sh4* - FW2 x RW2 sequence was similar to shattering 4 (*sh4*) genes in rice populations (Fig. 3a and b).

In the case of flag leaf cDNA of weedy rice, the results showed that the *qsh1* - FW2 x RW2 sequence was similar to *qsh1* gene for putative transcription factor *qsh1* of rice populations and mRNA of *Oryza sativa japonica* Group. (Fig. 4a and b) Table 4. gives the detailed results of the tBLASTx. The NCBI conserved Domain Search programme, also showed that the *qsh1* FW2 x RW2 sequence of amplicon at ~750bp belonged to homeobox kn domain (Fig. 5).

Clustal Phylogeny program was used to create phylogenetic tree and to show which genes were related to the nucleotide query. Phylogenetic tree constructed for *sh4* FW2 x RW2, showed that the weedy rice *sh4* FW2 x RW2 query sequence had highest similarity to shattering (*sh4*) genes of *Oryza rufipogon, Oryza sativa, Oryza meridionalis voucher* (Fig. 6). Phylogenetic tree constructed for *sh4* FW2 x RW2 query sequence was closely related to *qsh1* gene of *Oryza sativa indica* Group, *Oryza sativa japonica* Group and *Oryza rufipogon* (Fig. 7).

4.2 EXPRESSION STUDIES OF THE IDENTIFIED GENE

4.2.1 Semi Quantitative Analysis

Semi quantitative analysis was done by doing a PCR using cDNA from different tissues *viz.*, Weedy rice leaf (flag leaf), weedy rice stem, root and grains as template with *sh4* FW2 x RW2 and *qsh1* FW2 x RW2 primers and Actin primers.

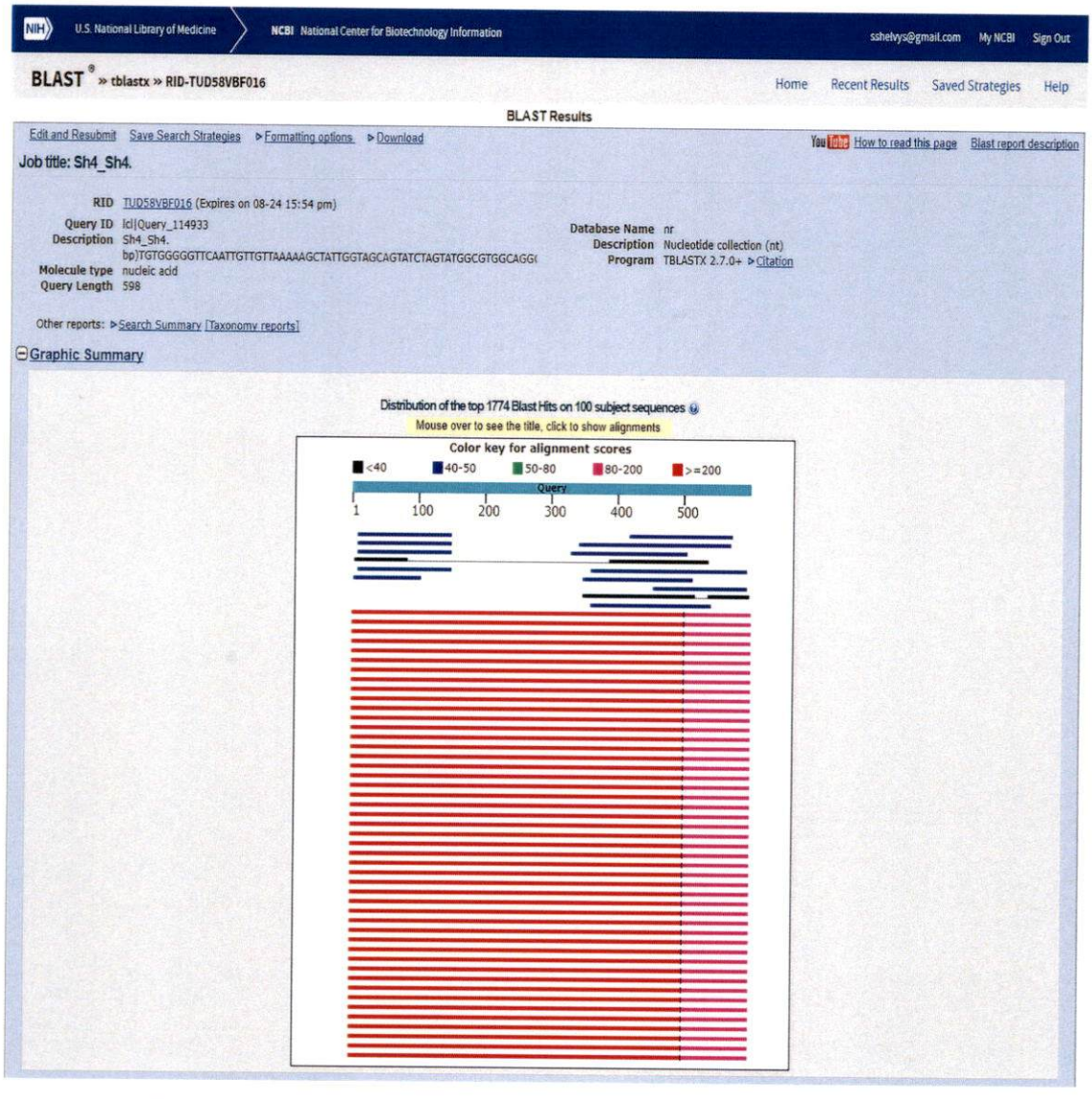


Figure 3a. Figure showing the result of tBlastx *sh4* FW2 x RW2

Sequences producing significant alignments:

Select: All None Selected: 0

Alignments | Download | GenBank Graphics

Description	Max score	Total score	Query cover	E value	N	Accession
Oryza sativa Indica Group cultivar Shuhui498 chromosome 4 sequence	323	2135	100%	6e-97	3	CP018160.1
Oryza sativa Indica Group cultivar RP Bio-226 chromosome 4 sequence	323	2135	100%	6e-97	3	CP012612.1
Oryza nivara voucher IRRI seed accession 81903 shattering 4 (sh4) gene, exon 1 and partial cds	323	2135	100%	6e-97	3	JN697603.1
Oryza sativa Japonica Group bio-material seed accession P9-1 shattering 4 (sh4) gene, exon 1 and partial cds	323	2135	100%	6e-97	3	JN697611.1
Oryza sativa Japonica Group bio-material seed accession P1-2 shattering 4 (sh4) gene, exon 1 and partial cds	323	2135	100%	6e-97	3	JN697609.1
Oryza nivara voucher IRRI seed accession 106431 shattering 4 (sh4) gene, exon 1 and partial cds	323	2135	100%	6e-97	3	JN697604.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2135	100%	6e-97	3	JN679472.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2135	100%	6e-97	3	JN679448.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2135	100%	6e-97	3	JN679323.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2135	100%	6e-97	3	JN679292.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2135	100%	6e-97	3	JN679268.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2135	100%	6e-97	3	JN679267.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2135	100%	6e-97	3	JN679250.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2135	100%	6e-97	3	JN679233.1
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Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679301.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679295.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679280.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679278.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679273.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679270.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679269.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679258.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679256.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679277.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679252.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679253.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679299.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679291.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679288.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2119	100%	2e-96	3	JN679306.1
Oryza sativa voucher 10A putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU220920.1
Oryza sativa Indica Group voucher RA4978 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU221033.1
Oryza sativa voucher 1210-02 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU220957.1
Oryza sativa voucher 1001-01 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU220932.1
Oryza sativa voucher 1004-01 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU220907.1
Oryza sativa Japonica Group voucher RA4988 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU221046.1
Oryza sativa Japonica Group voucher RA5294 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU221041.1
Oryza sativa Indica Group voucher RA5345 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU221025.1
Oryza sativa Indica Group voucher RA4979 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU221022.1
Oryza rufipogon voucher 100904 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU220981.1
Oryza sativa voucher 1996-08 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU220951.1
Oryza sativa voucher TX4 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU220931.1
Oryza sativa voucher 1096-01 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU220911.1
Oryza sativa voucher 1025-01 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU220908.1
Oryza sativa Indica Group voucher RA4974 putative shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	GU221032.1
Oryza nivara IRGC seed accession 106061 shattering 4 (sh4) gene, exon 1 and partial cds	323	2135	100%	6e-97	3	EU999944.1
Oryza nivara IRGC seed accession 105742b shattering 4 (sh4) gene, exon 1 and partial cds	323	2135	100%	6e-97	3	EU999943.1
Oryza nivara IRGC seed accession 103407b shattering 4 (sh4) gene, exon 1 and partial cds	323	2135	100%	6e-97	3	EU999934.1
Oryza sativa genomic DNA, chromosome 4, BAC clone: H0624F09, complete sequence	323	2135	100%	6e-97	3	AL732356.2
Oryza sativa (indica cultivar-group) cultivar CL 16 shattering protein (sh4) gene, complete cds	323	2135	100%	6e-97	3	DQ383373.1
Oryza sativa (indica cultivar-group) cultivar BL 272 shattering protein (sh4) gene, partial cds	323	2135	100%	6e-97	3	DQ383407.1
Oryza rufipogon voucher IRGC 80529 shattering protein (sh4) gene, partial cds	323	2135	100%	6e-97	3	DQ383398.1
Oryza nivara voucher IRGC 103838 shattering protein (sh4) gene, partial cds	323	2135	100%	6e-97	3	DQ383377.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2112	100%	2e-96	3	JN679304.1
Shattering 4 (sh4) gene, DNA linear exon 1 and partial cds	323	2104	100%	2e-96	3	JN679302.1
Oryza rufipogon voucher 105388 putative shattering protein (sh4) gene, complete cds	323	2119	100%	2e-96	3	GU220989.1
Oryza nivara IRGC seed accession 105734 shattering 4 (sh4) gene, exon 1 and partial cds	323	2119	100%	2e-96	3	EU999941.1
Oryza nivara IRGC seed accession 105319 shattering 4 (sh4) gene, exon 1 and partial cds	323	2119	100%	2e-96	3	EU999937.1

Figure 3b. Figure showing the result of tBlastx *sh4* FW2 x RW2

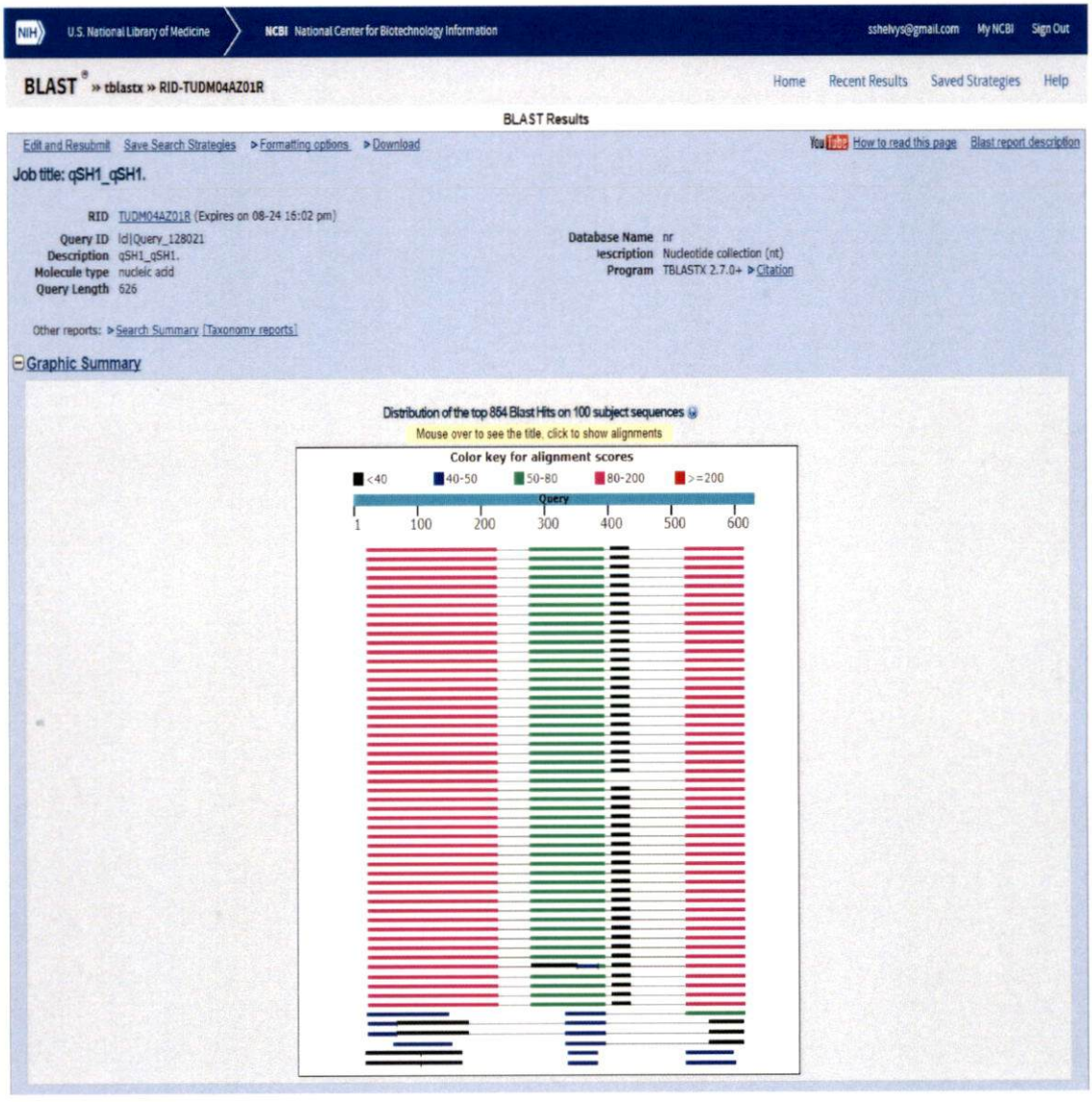

Figure 4a. Figure showing the result of tBlastx *qsh1* FW2 x RW2

uences producing significant alignments:

ct All None Selected:0

Alignments Download GenBank Graphics

Description	Max score	Total score	Query cover	E value	N	Accession
PREDICTED: Oryza sativa Japonica Group BEL1-like homeodomain protein 9 (LOC4324855), mRNA	127	894	70%	5e-55	3	XM_015786462.1
Oryza sativa Indica Group qSH1 gene for putative transcription factor qSH-1, complete cds, cultivar: 451	127	894	70%	1e-54	3	AB493306.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W1551	127	894	70%	1e-54	3	AB493325.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W630	127	894	70%	1e-54	3	AB493324.1
Oryza sativa Indica Group qSH1 gene for putative transcription factor qSH-1, complete cds, cultivar: 717	127	894	70%	1e-54	3	AB493309.1
Oryza sativa Indica Group qSH1 gene for putative transcription factor qSH-1, complete cds, cultivar: 108	127	894	70%	1e-54	3	AB493301.1
Oryza sativa Indica Group qSH1 gene for putative transcription factor qSH-1, complete cds, cultivar: 868	127	894	70%	1e-54	3	AB493303.1
Oryza sativa Indica Group qSH1 gene for putative transcription factor qSH-1, complete cds, cultivar: Kasalath	127	894	70%	1e-54	3	AB493307.1
Oryza sativa Indica Group qSH1 gene for putative transcription factor qSH-1, complete cds, cultivar: 414	127	894	70%	1e-54	3	AB493304.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W2005	127	847	70%	1e-54	3	AB493335.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: Acc105416	127	894	70%	1e-54	3	AB493327.1
Oryza sativa Japonica Group qSH1 gene for putative transcription factor qSH-1, complete cds, cultivar: 773	127	894	70%	1e-54	3	AB493288.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W1945	127	894	70%	1e-54	3	AB493343.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W1944	127	894	70%	1e-54	3	AB493342.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W1943	127	894	70%	1e-54	3	AB493341.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W120	127	894	70%	1e-54	3	AB493332.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W1807	127	894	70%	1e-54	3	AB493331.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W154	127	894	70%	1e-54	3	AB493330.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: Acc105451	127	894	70%	1e-54	3	AB493328.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W1990	127	894	70%	1e-54	3	AB493322.1
Oryza sativa Japonica Group qSH1 gene for putative transcription factor qSH-1, complete cds, cultivar: Nipponbare	127	894	70%	1e-54	3	AB493281.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W149	127	894	70%	1e-54	3	AB493333.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W2108	127	885	70%	1e-54	3	AB493348.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W2107	127	885	70%	1e-54	3	AB493347.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W1970	127	892	70%	4e-54	3	AB493346.1
Oryza rufipogon voucher IRGC 105400 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	869	66%	1e-54	3	EU006057.1
Oryza sativa voucher IRGC 25840 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	894	70%	1e-54	3	EU006045.1
Oryza nivara voucher IRGC 103834 putative transcription factor qSH-1-like (qSH-1) gene, complete sequence	127	894	70%	1e-54	3	EU006062.1
Oryza nivara voucher IRGC 103415 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	894	70%	1e-54	3	EU006060.1
Oryza sativa voucher IRGC 64793 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	894	70%	1e-54	3	EU006046.1
Oryza sativa Indica Group voucher IRGC 66513 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	894	70%	1e-54	3	EU006041.1
Oryza sativa Indica Group voucher IRGC 27342 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	894	70%	1e-54	3	EU006040.1
Oryza sativa voucher IRGC 27856 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	894	70%	1e-54	3	EU006049.1
Oryza nivara voucher IRGC 105695 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	894	70%	1e-54	3	EU006063.1
Oryza sativa voucher IRGC 13746 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	894	70%	1e-54	3	EU006047.1
Oryza rufipogon voucher IRGC 106430 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	894	70%	1e-54	3	EU006059.1
Oryza rufipogon voucher IRGC 106413 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	885	70%	1e-54	3	EU006058.1
Oryza rufipogon voucher IRGC 103847 putative transcription factor qSH-1 (qSH-1) gene, complete cds	127	887	70%	8e-54	3	EU006055.1
Oryza sativa Japonica Group qSH-1 mRNA for qSH-1, complete cds	127	894	70%	4e-55	3	AB071331.1
Oryza sativa Indica Group qSH-1 mRNA for qSH-1, complete cds	127	894	70%	4e-55	3	AB071330.1
Oryza sativa Indica Group qSH-1 gene for qSH-1, complete cds	127	894	70%	1e-54	3	AB071332.1
Oryza sativa Japonica Group qSH-1 gene for qSH-1, complete cds	127	894	70%	1e-54	3	AB071333.1
Oryza sativa Japonica Group genomic DNA, chromosome 1, clone:P0005H10	127	894	70%	1e-51	3	AP004127.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W2002	127	891	70%	1e-53	3	AB493323.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W2007	127	891	70%	1e-53	3	AB493336.1
Oryza rufipogon qSH1 gene for putative transcription factor qSH-1, complete cds, strain: W106	127	888	70%	6e-53	3	AB493320.1
Oryza sativa Indica Group cultivar Shuhui498 chromosome 1 sequence	127	894	70%	8e-47	3	CP018157.1
Oryza sativa Indica Group cultivar RP Bio-226 chromosome 1 sequence	127	894	70%	9e-47	3	CP012609.1
Oryza sativa Japonica Group DNA, chromosome 1, cultivar: Nipponbare, complete sequence	127	894	70%	8e-47	3	AP014957.1
Oryza sativa Japonica Group qSH1 gene for putative transcription factor qSH-1, complete cds, cultivar: 775	125	849	70%	5e-54	3	AB493289.1
PREDICTED: Oryza brachyantha BEL1-like homeodomain protein 9 (LOC102715365), partial mRNA	74.4	228	24%	5e-17	2	XM_015832664.1
PREDICTED: Aegilops tauschii subsp. tauschii BEL1-like homeodomain protein 9 (LOC109760972), mRNA	45.5	298	43%	3e-11	3	XM_020319755.1
Triticum aestivum cDNA, clone: WT008_K08, cultivar: Chinese Spring	45.5	298	43%	2e-11	3	AK333815.1
Triticum aestivum WBLH4 mRNA for BEL1-type homeodomain protein, complete cds	45.5	149	18%	1e-04	2	AB546647.1
Alloactinosynnema sp. L-07 genome assembly Alloactinosynnema sp. L-07, chromosome : I	45.1	45.1	20%	0.38	1	LN850107.1
PREDICTED: Elaeis guineensis BEL1-like homeodomain protein 9 (LOC105046344), mRNA	44.6	85.1	7%	0.53	1	XM_010924900.2
PREDICTED: Phoenix dactylifera BEL1-like homeodomain protein 9 (LOC103715490), mRNA	43.2	43.2	7%	1.4	1	XM_008803132.2
PREDICTED: Brachypodium distachyon BEL1-like homeodomain protein 9 (LOC100823746), mRNA	43.2	218	16%	0.001	2	XM_003564561.3
PREDICTED: Ananas comosus BEL1-like homeodomain protein 9 (LOC109728198), mRNA	43.2	43.2	7%	1.4	1	XM_020258557.1

Figure 4b. Figure showing the result of tBlastx *qsh1* FW2 x RW2

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Table 4. tBLASTx results of the sequenced fragments

cDNA samples	**Sequence identifier**	**tBLASTx hits**	**Gene**
Flag leaf of weedy rice	*sh4* FW2 x RW2	1774 Blast Hits	Shattering 4 (*sh4*) gene
Flag leaf of weedy rice	*qsh1* FW2 x RW2	854 Blast Hits	Putative transcription factor *qsh1* gene

Figure 5. Figure showing the result of NCBI conserved Domain Search programme

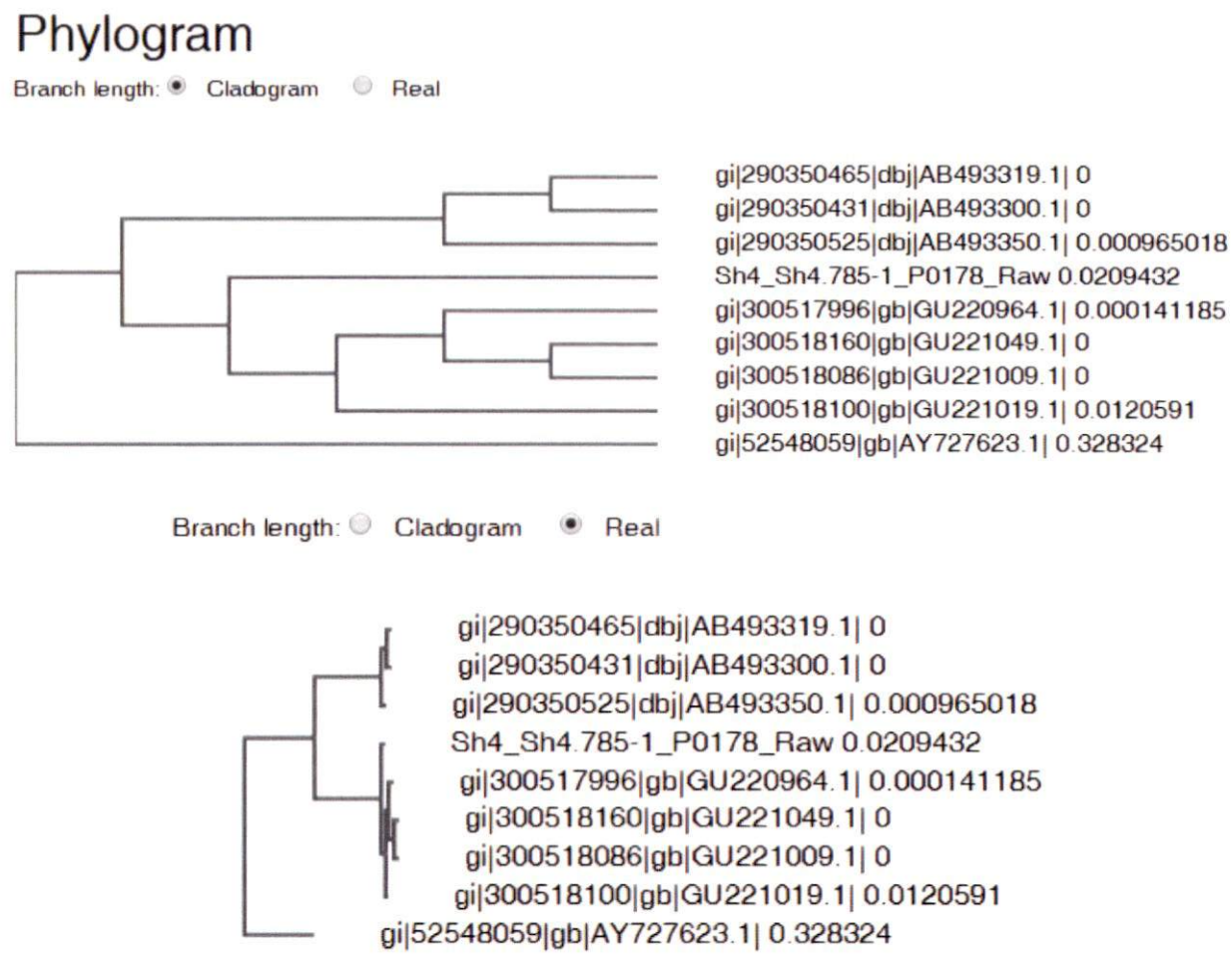


Figure 6. Phylogenetic tree created using Clustal Phylogeny program of *sh4* FW2 x RW2 sequence

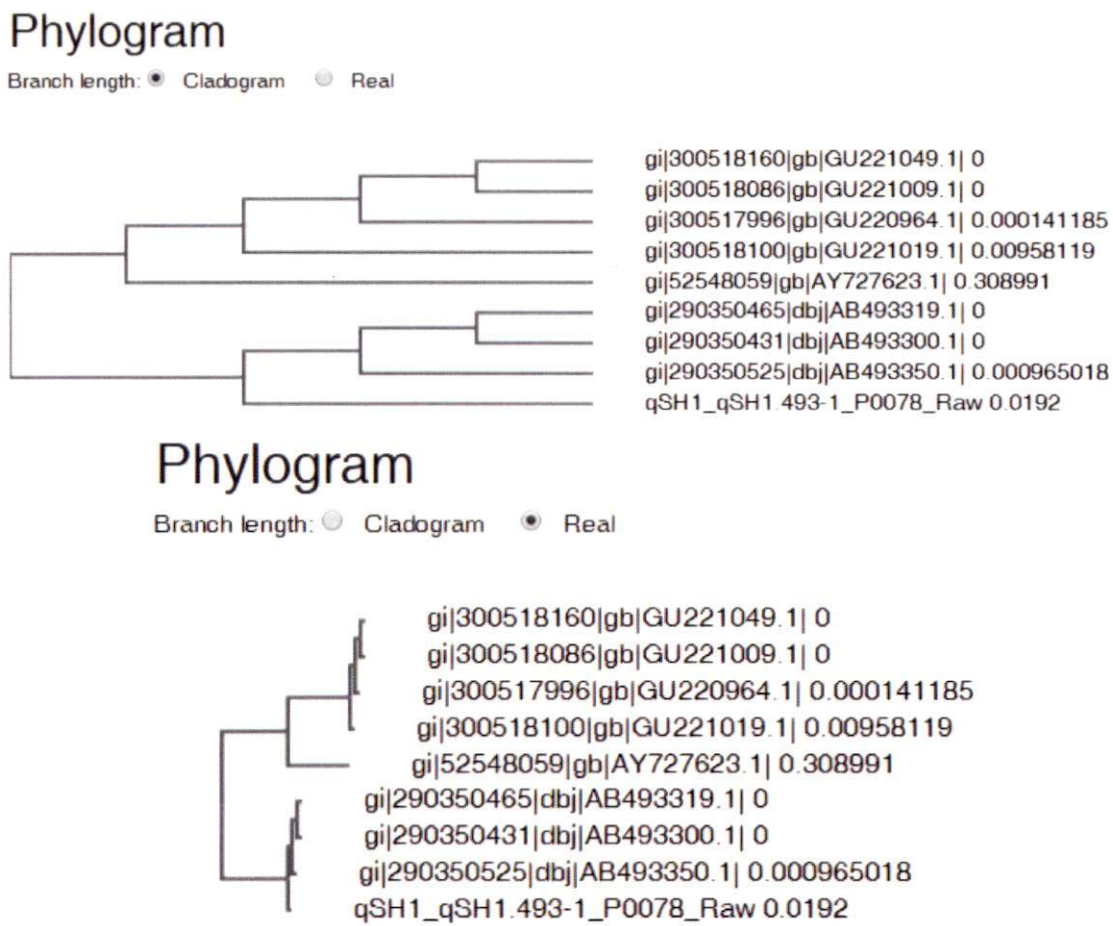


Figure 7. Phylogenetic tree created using Clustal Phylogeny program of *qsh1* FW2 x RW2 sequence

Table 5. Actin primers used for semi quantitative analysis

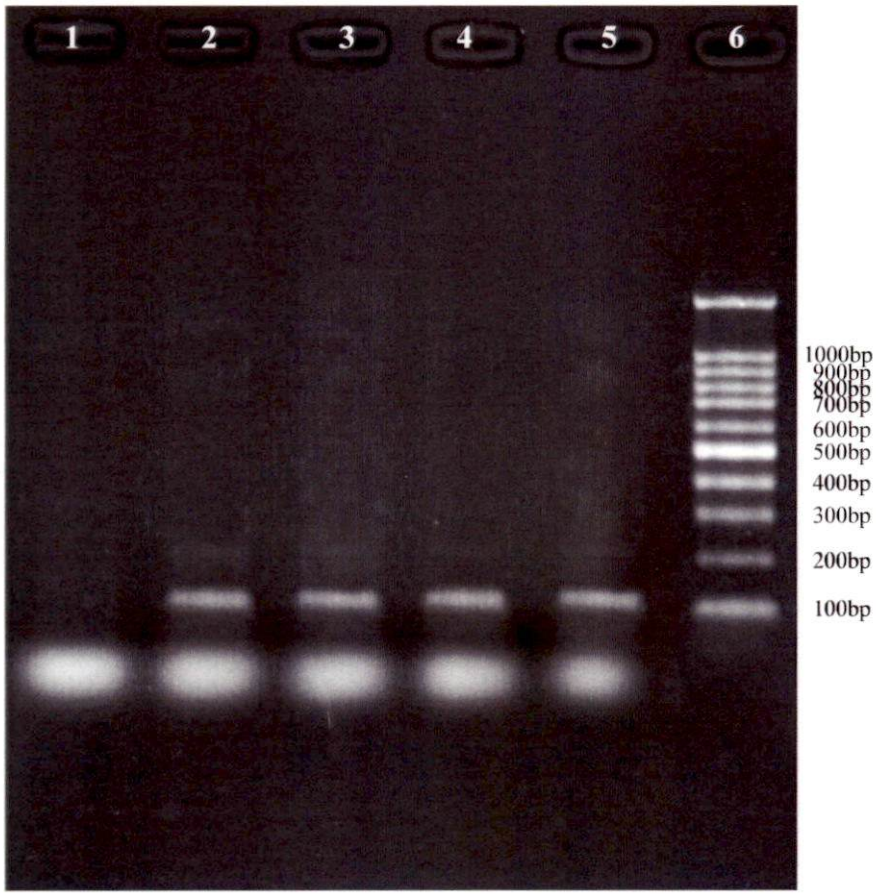
Sl. No.	Target gene	Primer name	Primer sequence (5' to 3')	No. of bases	GC content (%)	T_m (°C)
1	ACTIN	*ACT*- F	CTGGTGATGGTGTGAGCCAC	20	60	62.5
		ACT - R	CATGAAATAGCTGCGAAACG	20	45	56.4

The PCR products were separated on agarose gel (1.5%) and the gel was observed using gel documentation system. Table 5 shows the sequence of the Actin primers used in semi quantitative analysis. The gel pictures of PCR products with Actin primers showed that there was amplification in all the tissues (Plate 9).

Gel pictures of PCR products of the *sh4* FW2 x RW2 primer showed no amplification in any of the tissues except weedy rice leaf (flag leaf) and grains with an amplification at ~650bp of the expected amplicon (Plate 10).

Also, when *qsh1* FW2 x RW2 degenerate primers were used, gel pictures of PCR products (Plate 10) showed that there was no amplification in all tissues except weedy rice leaf (flag leaf) at ~750bp.

09



Legends

1 - Control
2 - Weedy rice root
3 - Weedy rice grain
4 - Weedy rice culm
5 - Weedy rice leaf (Flag leaf)
6 - 100bp ladder

Plate 13. Agarose gel electrophoresis of PCR product of cDNA from different tissues of weedy rice with Actin primer

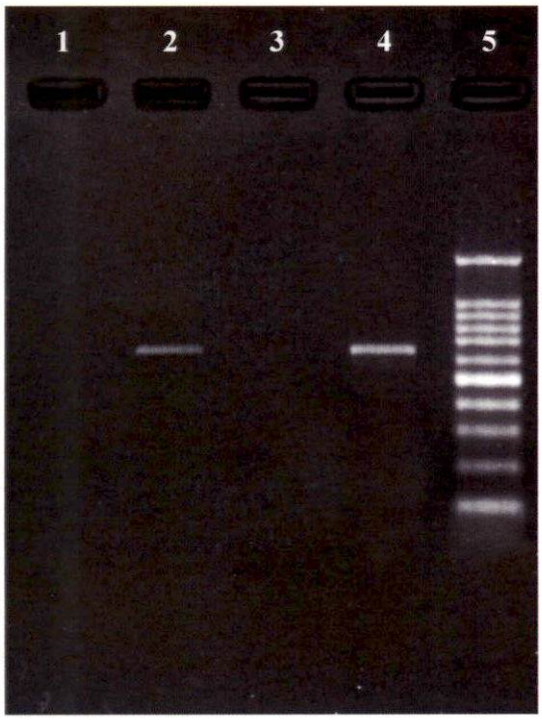


Plate 14. Agarose gel electrophoresis of PCR product of cDNA from different tissues of weedy rice with *sh4* FW2 x RW2 primers

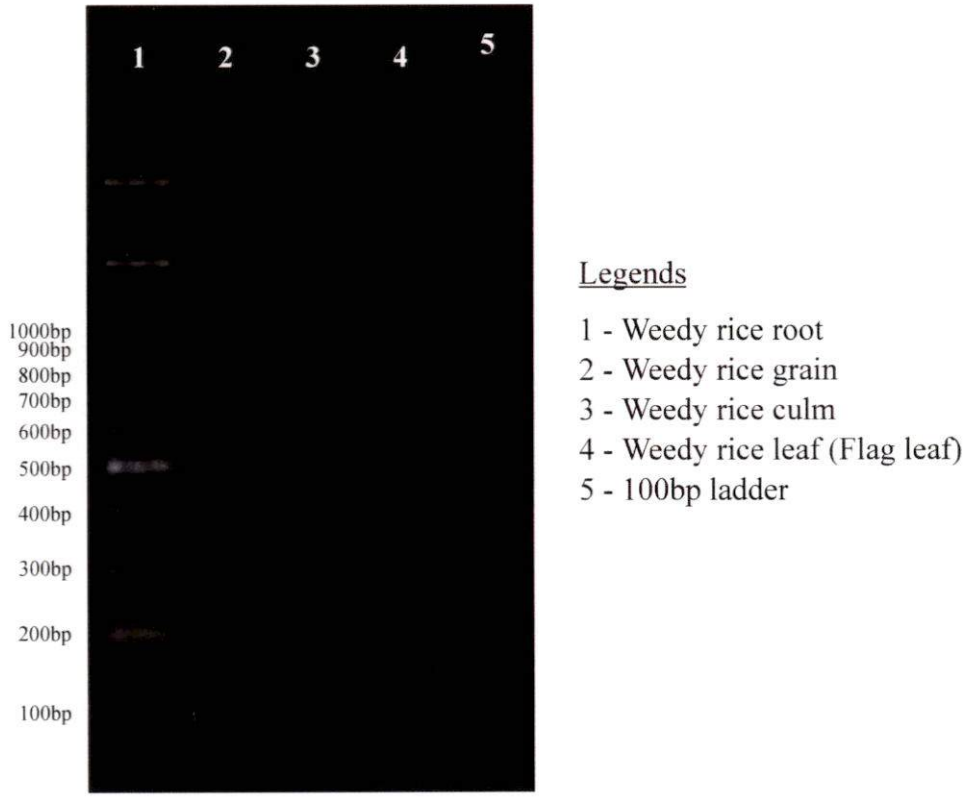


Plate 15. Agarose gel electrophoresis of PCR product of cDNA from different tissues of weedy rice with *qsh1* FW2 x RW2 primers

Discussion

5. DISCUSSION

Weedy rice (*Oryza sativa* f. *spontanea*) is the complex of morphotypes of *Oryza* species widely distributed in the commercial rice fields in more than 50 countries of Asia, Africa and Latin America, especially in areas where farmers have switched to direct seeding due to labour shortage and high cost (Abraham and Jose, 2014). According to Prathepha (2011), weedy rice is highly characterized by its seed shattering, which apparently increases the distribution of this species. Genes like *sh4* (grain shattering quantitative trait locus on chromosome 4) and *qsh1* (quantitative trait locus of seed shattering on chromosome 1) have been identified as required for reduced seed shattering during rice (*Oryza sativa*) domestication (Zhou *et al.*, 2012). Thus studying the genes involved in shattering is an initial step in employing molecular techniques to manipulate shattering. Isolation of the key genes in shattering was carried out in this study entitled 'Molecular characterization of shattering in weedy rice (*Oryza sativa* f. *spontanea*) biotypes of Kerala', in the Department of Plant Biotechnology and Department of Agronomy, College of Agriculture, Vellayani, Thiruvananthapuram, The results of the study are discussed in this chapter.

5.1 ISOLATION OF GENES RELATED TO SHATTERING

5.1.1 DNA Extraction and PCR Analysis

According to literature survey, it was found that two major genes responsible for shattering in weedy rice are *sh4* and *qsh1*. Based on *sh4* and *qsh1* sequences of other rice populations, degenerate primers were designed. Degenerate primers were prefered since they can amplify the weakly conserved sequences of the homologous genes in different organisms effectively (Shen *et al.*, 1998). These primers were used for PCR and RT-PCR analysis of genomic DNA and mRNA samples to amplify and isolate the orthologues of *sh4* and *qsh1* genes in weedy rice.

Two methods were adopted for isolating DNA from leaf and seeds, because isolation of DNA from seeds are more difficult than other tissues. A single DNA isolation protocol is not likely to be applicable for all plant systems (Almakarem *et al.*, 2012).

Modified SDS method of genomic DNA standardized by Dellaporta *et al.* (1983) was used for extraction of genomic DNA from flag leaf of both weedy rice and cultivated rice variety *Uma,* as it has been mostly accepted in rice species (Aliyu *et al.,* 2013) . In SDS method of DNA extraction, the plant cell wall was broken using mechanical force in the presence of extraction buffer. SDS in the buffer which liberated DNA by lysing cell and nuclei (Manak, 1993). Subsequent centrifugation co-precipitated cell debris with polysaccharides and protein complexes, that interfere with the quality of the DNA. Tannins and other polyphenolics from the tissues were removed by adding high concentrations of PVP and ß-mercaptoethanol (Warude *et al.,* 2003).

For the extraction of genomic DNA from panicles of both weedy rice and cultivated rice variety *Uma,* CTAB method of DNA extraction method developed by Liang *et al.* (2015) was used. Extraction of DNA from seeds has two advantages. First, part of the endosperm in rice is sufficient for DNA isolation, leaving the corresponding viable embryo available for plant growth and phenotyping, which is important in Marker Assisted Selection (MAS) programmes. Second, isolating DNA from seeds accelerates genotyping by eliminating plant germination and growth (Mace *et al.,* 2003). The main disadvantage is, in comparison with leaf-based DNA isolation methods, seed-based DNA isolation methods in rice were rather limited due to the presence of seed reserves stored including lipids, carbohydrates, proteins and polyphenols (Komori *et al.,* 2004; Mutou *et al.*, 2014). The critical component of this CTAB method is α-Amylase, as it significantly improves the quality of the isolated rice DNA. Contamination with high concentrations of polysaccharide, which is an inhibitor of the enzymes in the case of downstream processes such as PCR, was a big problem with many seed DNA extraction methods. α-Amylase has proven to be efficacious and was used commercially to remove polysaccharide contamination and increase PCR amplification rates in DNA extraction (Anon., 2015).

According to Weising *et al.* (2005), the absorbance ratio (A_{260}/A_{280}) in the range of 1.8 to 2.0 indicates a high level of purity of DNA. The isolated genomic DNA from weedy rice tissues showed high purity with (A_{260}/A_{280}) value ranging from 1.76 to 1.83 in this study.

Initially, PCR amplification using isolated genomic DNA from both weedy rice and *Uma* leaves were used with the different sets of designed degenerate primers. Two of the *sh4* primers and one of the *qsh1* primer designed for shattering yielded desired amplification at the expected size of ~649bp, ~696bp and ~747bp respectively. When the genomic DNA of panicles of both weedy rice and variety *Uma* were used as templates for the PCR amplification, same result was observed. Hence it was assumed that the degenerate primers designed are functional and both *sh4* and *qsh1* genes are present in weedy rice and variety *Uma*.

5.1.2 RNA Isolation and RT-PCR Analysis

At least two loci *sh4* and *qsh1* of large effect influencing degree of shattering have been cloned in cultivated rice, *sh4* (Li *et al.*, 2006) and *qsh1* (Konishi *et al.*, 2006), yet nothing is known about the weedy rice alleles at these loci. Therefore, the isolation of RNA was done from both weedy rice and cultivated rice variety *Uma* from leaf and culm at seedling, tillering, and flowering stages.

The quality, quantity and integrity of the RNA recovered is very important for successful RNA extraction. Hence, Both CTAB and TRIZOL methods were used initially for extraction of high quality total RNA. After checking the quality it was observed that CTAB method yielded high integrity total RNA. Total RNA isolated from CTAB method was used for further analysis. According to Yang *et al.* (2008), a modified protocol using CTAB as the cell disrupting agent with the addition of β - mercaptoethanol as a reducing agent, prevents oxidation of polyphenols and inhibit RNase activity. Lithium chloride (LiCl) was used to precipitate RNA. To minimize RNase contamination proper aseptic techniques needed to be strictly employed, by allowing the use of sterile materials, which determines the quality of the isolated RNA (MacRae, 2007). The bands in agarose gel showed clear and discrete ribosomal RNAs confirming good quality.

The absorbance ratio (A_{260}/A_{280}) in the range of 1.7 to 2.0 indicated a high level of purity of RNA without any contamination of DNA or protein (Accerbi *et al.*, 2010). The A_{260}/A_{280} values obtained in this study ranging from 1.77 to 2.13 revealed that the extracted RNA was of good quality except that of culm at tillering stage

of weedy rice and *Uma* variety where the ratios were 2.21 and 1.47 respectively. RT-PCR amplification of the extracted RNA was done using the set of designed degenerate primers at specific annealing temperatures after the cDNA synthesis. The synthesis of cDNA was done using Oligo$(dT)_{23}$ primer with the RNA samples of both weedy rice and cultivated rice variety *Uma* from leaf and culm at seedling, tillering, and flowering stages. According to Nam *et al.* (2002), Oligo(dT) primer is widely used for cDNA synthesis, to generate a high frequency of truncated cDNAs through internal poly (A) priming. In the current dbEST databases, such truncated cDNAs may contribute to 12% of the expressed sequence tags . Degenerate *sh4* and *qsh1* primer set of *sh4* FW2 x RW2 and *qsh1* FW2 and RW2 produced amplification in flag leaf in weedy rice only, while there was no amplification observed in all other cDNA samples including cultivated variety *Uma.*

The primer set *sh4* FW2 x RW2 did produce an amplification with an amplicon size of ~650bp and the primer set *qsh1* FW2 x RW2 did produce an amplification with an amplicon size of ~750bp only in the flag leaf of weedy rice. They yielded expected size amplicons. But no amplification was observed in other cDNA samples including tissues from cultivated variety *Uma.* The result clearly revealed that the gene responsible for shattering viz., *sh4* and *qsh1* are present in both weedy rice and cultivated variety *Uma* as evidence from the PCR amplification of genomic DNA. However, the genes responsible for shattering investigated in the present study viz., *sh4* and *qsh1* are not expressed in any of the growth stages in cultivated rice variety *Uma.* The reasons behind may be the gene is not expressed or poor mRNA stability, promoter mutations, truncation etc. According to Purugganan and Fuller (2009), nearly all weedy rice population shatters its seeds while cultivated rice does not, as they have been selected to retain their seeds during the domestication process to make them easier to harvest. Konishi *et al.* (2006) reported that a single nucleotide polymorphism (SNP) in the 59 upstream regulatory region of *qsh1* causes *qsh1* expression to disappear from the abscission layer, thus leading to a decline in seed shattering over the history of rice domestication (Ishikawa *et al.,* 2010). According to Zhu *et al.* (2012) during the early rice domestication the initial

origin of non -shattering involved a single gene of large effect, specifically, the *sh4* locus, with the evolutionary replacement of a dominant allele for shattering with a recessive allele for non-shattering. Most published sequence data of the *sh4* locus are reliable with that speculation including the replacement of the wild type, with the seed-shattering "G" nucleotide, by the mutational type, with non-shattering "T" nucleotide, at *sh4*'s functional nucleotide polymorphism (FNP) site as the key event in the domestication process of Asian cultivated rice.

5.1.3 Sequencing and Sequence Analysis

The bands obtained in all the reactions mentioned above, including those at the expected sizes (~650bp and ~750bp), were eluted, purified and sequenced. The sequences of the amplicons were analyzed using bioinformatic tools in order to identify the gene *viz*., Sequence Manipulation Suite (Stothard, 2000), tBLASTx (Altschul, 1997) and Clustal Phylogeny program(Saitou and Nei, 1987).

The tBLASTx results showed that *sh4* FW2 x RW2 sequence of amplicon size ~650bp (obtained by reaction using weedy rice flag leaf cDNA) belonged to mRNA of Shattering 4 (*sh4*) genes among various rice populations. Examination of *sh4* alleles has shown that all cultivated rice sampled to date shares the non-shattering T mutation, and most rice individuals share a common *sh4* haplotype, despite the fact that at least two separate domestication events gave rise to cultivated Asian rice (Li *et al.,* 2006b; Zhang *et al.,* 2009).

The *qsh1* locus is a homeodomain gene, similar to *Arabidopsis thaliana* REPLUMLESS, which was isolated in a cross between two *Orysa sativa* varieties, aus and temperate *japonica*, that differ in their shattering propensity. Variations in seed shattering between *indica* and *japonica* are controlled by five QTLs. They are *qsh11*, *qsh12* in *indica*, and *qsh1*, *qsh2* and *qsh5* in *japonica*. All these contribute to a reduction in seed shattering by preventing abscission layer formation. The *qsh1* QTL has the greatest effect as it explains 68.6% of the total phenotypic variation in the population. At the same time, *qsh1* reduces the expression of the transcription factor only at the provisional abscission layer, which results in decreased seed

shattering (Konishi *et al.,* 2006). In this study the tBLASTx results showed that *qsh1* FW2 x RW2 sequence of amplicon at ~750bp (obtained by reaction using weedy rice flag leaf cDNA) belonged to mRNA of *Oryza sativa japonica* group BEL1-like homeodomain protein 9 (*Accession No.* XM_015786462.1). The NCBI conserved Domain Search programme, showed that the *qsh1* FW2 x RW2 sequence of amplicon at ~750bp (obtained by reaction using weedy rice flag leaf cDNA) belonged to homeobox kn domain. Hence this is the first ever *qsh1* gene in *Orysa sativa f. spontanea* to be identified and sequenced.

In the case of rice domestication previous phylogenetic analyses based on a variety of molecular markers have shown that *indica* and *japonica* cultivars fall into seperate clades, thus supporting independent domestication of the two cultivars (Second, 1982; Cheng *et al.,* 2003; Zhu and Ge, 2005; Londo *et al.,* 2006). According to Ferrero (2010), the phylogenetic starting point of the weedy structures is firmly identified with that of cultivated rice. However, here phylogenetic trees were created using Clustal Phylogeny. Phylogenetic tree constructed for *sh4* FW2 x RW2 sequence, had highest similarity to shattering (*sh4*) gene of *Oryza meridionalis* voucher and also had relationship with *Oryza rufipogon* and *Oryza sativa.* Moreover, although cultivated and weedy rice groups differ greatly in their shattering ability, all sampled weedy and domesticated accessions possess similar or identical alleles at the *sh4* locus, suggesting that the domestication and associated substitution at *sh4* is not sufficient for loss of shattering (Sang and Ge, 2007).

In the Phylogenetic tree constructed for the weedy rice *qsh1* FW2 x RW2 query sequence was closely related to *qsh1* gene of *Oryza rufipogon,* together with *Oryza sativa indica* group and *Oryza sativa japonica* group. Thurber *et al.* (2010) reported that all US weedy rice individuals possess the ancestral allele of *qsh1* that is common in all non-temperate *japonica* cultivated and wild rice (*Orysa rufipogon*). The shattering associated single nucleotide polymorphism (SNP) at *qsh1* has not played a role in the evolution of weedy rice, as all weeds, wild rice, and most cultivars share the ancestral allele at this locus.

5.2 EXPRESSION STUDIES OF THE IDENTIFIED GENE IN WEEDY RICE

5.2.1 Semi Quantitative Analysis

Semi quantitative analysis was done by performing a PCR using cDNA from different tissues of Weedy rice *viz*., leaf (flag leaf), stem, root and grain as template with *sh4* FW2 x RW2 and *qsh1* FW2 x RW2 primers and Actin was used as a reference gene to check samples. The actin gene encodes a structural protein of cytoskeleton, and is perhaps the most widely used gene for normalization in the experiments of gene expression (Pohjanvirta *et al.,* 2006). Several authors confirmed that the actin used as housekeeping presented differential expression in various tissues (Selvey *et al.,* 2001; Barber *et al.,* 2005). PCR product with Actin primers showed amplification in all the wells except in control reaction, without template cDNA. This is because Actin is a housekeeping gene which is expressed in all the stages of the plant and it also proved that the cDNA from different tissues was functional.

When *sh4* FW2 x RW2 primers were used to study the expression of weedy rice *sh4* gene, no amplification was observed in any of the tissues, except in flag leaf and grain. This may be because the *sh4* gene is active in the flag leaf and grains, all others such as root and culm is relatively inactive. In the case of *qsh1* FW2 x RW2 primers, there was amplification only in flowering stage of the plant in flag leaf and other plant parts did not produce any amplification. The above genes are not expressed in other plant parts which can be due to the facts like poor mRNA stability, promoter mutations, truncation etc.

From this study it can be inferred that the genes responsible for shattering in weedy rice biotypes of Kerala viz. *sh4* and *qsh1* are present both in cultivated rice and weedy rice. However, the genes are expressed only in weedy rice during the flowering stage.

SUMMARY

6. SUMMARY

The study entitled "Molecular characterization of shattering in weedy rice (*Oryza sativa* f. *spontanea*) biotypes of Kerala" was conducted at the Integrated Biotechnology Block, College of Agriculture, Vellayani, Thiruvananthapuram during 2015-2017. The objective of the study was isolation and sequencing of genes related to shattering viz. *sh4* and *qsh1* in weedy rice biotypes and characterization by expression profiling and phylogenetic analysis.

Degenerate primers were designed for *sh4* and *qsh1* genes based on the gene sequences retrieved from NCBI database (*sh4* and *qsh1* forward and reverse primers) which were used to isolate and identify the genes. Both genomic DNA and Total RNA of weedy rice and cultivated rice *Uma* were isolated. Followed by synthesis of cDNA using M-MuLV (Moloney murine leukaemia virus) Reverse Transcriptase and Oligo dT primers, PCR (Polymerase chain reaction) was done using both genomic DNA and cDNA. Amplification was obtained in two sets of designed primers *i.e. sh4* (FW2 x RW2) and *qsh1* (FW2 x RW2). When DNA was used as template for PCR, two of the *sh4* primers and one of the *qsh1* primers designed for shattering yielded amplificons at the expected size of 649bp, 696bp and 747bp respectively. PCR in cDNA samples of RNA amplicon of size ~690bp with the primer combination of *sh4* (FW2 x RW2) in flag leaf was observed. Also, an amplicon of size ~750bp in flag leaf, cDNA was produced using degenerate *qsh1* FW2 x RW2 primers. None of these primers produced an amplification in any of the cDNA samples of cultivated rice *Uma*.

Both the two bands obtained from cDNA were eluted, purified and sequenced. The sequences of the amplicon were analyzed using bioinformatics tools inorder to identify the gene *viz*., tBLASTx, NCBI conserved Domain Search and phylogenetic tree construction using Clustal Phylogeny program.

The tBLASTx programme showed that the amplicon of size ~690bp was similar to shattering 4 (*sh4*) genes of *Oryza* populations, whereas the amplicon of size ~750bp belonged to *qsh1* gene for putative transcription factor *qsh1* of rice populations and mRNA of *Oryza sativa japonica* group.

The NCBI conserved Domain Search programme, showed that the *qsh1* FW2 x RW2 sequence of amplicon at ~750bp (obtained by reaction using weedy rice flag leaf cDNA) belonged to homeobox kn domain. The sequence of the amplicons of *qsh1* obtained was the partial fragments of the first ever *qsh1* gene for putative transcription factor *qsh1* to be isolated from *Oryza sativa* f. *spontanea.* Phylogenetic tree constructed for *sh4* FW2 x RW2 sequence, had highest similarity to shattering (*sh4*) gene of *Oryza meridionalis* voucher and also had relationship with *Oryza rufipogon* and *Oryza sativa.* However, the weedy rice *qsh1* FW2 x RW2 query sequence was closely related to *qsh1* gene of *Oryza rufipogon,* together with *Oryza sativa indica* group and *Oryza sativa japonica* group. Semi quantitative analysis with *sh4* and *qsh1* gene primers showed that the *sh4* gene was expressed in flag leaf and grains and *qsh1* gene was expressed only in the flag leaf.

Confirmation of the functional roles of *qsh1* and *sh4* in weedy rice has to be done by complete sequencing (full-length gene) and other genes responsible for shattering in weedy rice has to be identified. The possibility of gene silencing to modify weedy rice biotypes into non - shattering ones has to be explored for its utilization as medicinal rice varieties may be undertaken as a future line of work.

References

7. References

[Anonymous]. 2015. *Example use of α-Amylase Ultrapure* [on line]. Toyama: Nippon Gene; c2015. Available:http://nippongene.com/siyaku/product/extraction/a-amylase ultrapure/a-amylase-ultrapure.html [15 August 2017].

Abraham, C. T. and Jose, N. 2014. Weedy rice invasion in rice fields of India and management options. *J. Crop Weed* 10(2): 365-374.

Abraham, C. T., Jose, N., and Rathore, M. 2012. Current status of weedy rice in India and strategies for its management. In: *Biennial Conference of ISWS on Weed Threat to Agriculture, Biodiversity and Environment*; 19-20, April, 2012, Thrissur. Kerala Agricultural University, Thrissur, p.8.

Accerbi, M., Schmidt, S. A., De Paoli, E., Park, S., Jeong, D. H., Pamela, J., and Green, P. J. 2010. Methods for isolation of total RNA to recover miRNAs and other small RNAs from diverse species. *Methods Mol. Biol.* 592: 31-50.

Aggarval, R. K., Brar, D. S., and Klush, G. S. 1997. Two new genomes in the Oryza complex identified on the basis of molecular divergence analysis, using total genomic DNA hybridization. *Mol. Gen. Genet.* 254: 1-12.

Aliyu, R. E., Wasiu, A. A., Dangora, D. B., Ademola, J. O., and Adamu, A. K. 2013. Comparative study of genomic DNA extraction protocols in rice species. *Int. J. Advmt. Res. Technol.* 2(2): 39-52.

Almakarem, A. S., Heilman, K. L., Conger, H. L., Shtarkman, Y. M., and Rogers, S. O. 2012. Extraction of DNA from plant and fungus tissues in situ. *BMC Res. Notes*. 5: 266-278.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 25(17): 3389-3402.

Azmi, M. and Karim, S. M. R. 2008. *Weedy Rice - Biology, Ecology and Management.* MARDI , Kuala Lumpur, Malaysia: Malaysian Agricultural Research and Development Institute (MARDI), 56p.

Baldi, G. 1971. Presenza del carattere pericarpo rosso in varietà di riso coltivate (*O. sativa*). *Il riso* 20: 299-302.

Barber, R. D., Harmer, D. W., Coleman, R. A., and Clark, B. J. 2005. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol. Genomics* 21: 389-395.

Basu, C., Halfhill, M. D., Mueller, T. C., and Stewart, C. N. 2004. Weed genomics: new tools to understand weed biology. *Trends Plant Sci.* 9: 391-398.

Cai, H. W. and Morishima, H. 2000. Genomic regions affecting seed shattering and seed dormancy in rice. *Theor. Appl. Genet.* 100: 840-846.

Chauhan, B. S. 2013. Effect of weedy rice variants and weedy rice density on the growth and yield of two rice cultivars in the Philippines. *Can. J. Plant Prot.* 1: 28-34.

Chen, L. J., Lee, D. S., Song, Z. P., Suh, H. S., and Lu, B. R. 2004. Gene flow from cultivated rice (*Oryza sativa*) to its weedy and wild relatives. *Ann. Bot.* 93: 67-73.

Cheng, C., Motohashi, R., Tsuchimoto, S., Fukuta, Y., Ohtsubo, H., and Ohstubo, E. 2003. Polyphyletic origin of cultivated rice: based on the interspersion pattern of SINEs. *Mol. Biol. Evol.* 20: 67-75.

Cohn, M. A. and Hughes, J. A. 1981. Seed dormancy in red rice (*Oryza sativa* L.) I. Effect of temperature on dry after-ripening red rice. *Weed Sci.* 29: 402-404.

Delatorre, C. A. 1999. Seed dormancy in red rice. *Ciencia Rural* 29: 565-571.

Dellaporta, S. L., Wood, J., and Hicks, J. B. 1983. A plant DNA minipreparation: Version II. *Plant Mol. Biol. Reporter* 1(4): 19-21.

Delouche, J. C., Burgos, N., Gaely, D., Zorrilla, G., and Labrada, R. 2007. *Weedy rices - origin, biology, ecology and control*, FAO Plant Production and Protection Paper 188, Rome, pp.3-15.

Diarra, A. R. J., Smith, R. J., and Talbert, R. E. 1985. Growth and morphological characteristics of red rice (*Oryza sativa*) biotypes. *Weed Sci.* 33: 310-314.

Do Lago, A. A. 1982. Characterization of red rice (*Oryza sativa* L.) phenotypes in Mississippi. Ph. D. dissertation, Mississippi State University, USA, 143p.

FAO (Food and Agriculture Organization) 1999. *Report of the Global workshop on red rice control.* Varadero, Cuba, 55p.

Federici, M. T., Vaughan, D., Tomooka, N., Kaga, A., Wang, X. W., Doi, K., Francis, M., Zorrilla, G., and Saldain, N. 2001. Analysis of Uruguayan weedy rice genetic diversity using AFLP molecular markers. *Electr. J. Biotechnol.* [e-journal] 4(3). available: http://www.ejbiotechnology.info/index.php/ejbiotechnology/article/view/465. ISSN 0717-3458 [02 January 2017].

Ferrero, A. 2010. Weedy rice, biological features and control. In: *Weed Management for Developing Countries. FAO of the United Nations,* Rome [on-line]. Available: http://www.fao.org/docrep/006/y5031e/y5031e09.htm [10 january 2017].

Fuller, D. Q., Qin, L., Zheng, Y. F., Zhao, Z. J., Chen, X., Hosoya, L. A., and Sun, G. P. 2009. The domestication process and domestication rate in rice: spikelet bases from the lower yangtze. *Sci.* 323: 1607-1610.

Gealy, D. R., Saldain, N. E., and Talbert, R. E. 2000. Emergence of red rice (*Oryza sativa*) ecotypes under dry-seeded rice (*Oryza sativa*) culture. *Weed Tech.* 14: 406-412.

Green, J. M., Barker, J. H. A., Marshall, E. J. P., Froud-Williams, R. J., Peters, N. C. B., Arnold, G. M., Dawson, K., and Karp, A. 2001. Microsatellite analysis of the inbreeding grass weed Barren Brome (*Anisantha sterilis*) reveals genetic diversity at the within- and between- farm scales. *Mol. Ecol.* 10: 1035-1045.

Harlan J. R. 1992. *Crops and Man* (2nd Ed.). WI: American Society of Agronomy, Madison, 284p.

Harlan, J. R. 1975. Geographic patterns of variation in some cultivated plants. *J. Heredity.* 66: 182-191.

He, Q., Kim, K. W., and Park, Y. J. 2017. Population genomics identifies the origin and signatures of selection of Korean weedy rice. *Plant Biotechnol. j.* 15(3): 357-366.

He, Z., Jiang, X., Ratnasekera, D., Grassi, F., Perera, U., and Lu, B. R. 2014. Seed mediated gene flow promotes within- population genetic variation of weedy rice: implication for weed management. *PLOS One* 9(12): 112-228.

IRGSP (International Rice Genome Sequencing Project) 2005. The map based sequence of the rice genome. *Nature* 436: 793-800.

Ishikawa, R., Thanh, P. T., Nimura, N., Htun, T. M., Yamasaki, M., and Ishii, T. 2010. Allelic interaction at seed-shattering loci in the genetic backgrounds of wild and cultivated rice species. *Genes Genet. Syst.* 85(4): 265-271.

Izawa, T. 2008. The process of rice domestication: a new model based on recent data. *Rice* 1(2): 127-134.

Ji, H. S., Chu, S. H., Jiang, W., Cho, Y. I., Hahn, J. H., Eun, M. Y., Mc Couch, S. R., and Koh, H. J. 2006. Characterization and mapping of a shattering mutant in rice that corresponds to a block of domestication genes. *Genet.* 173(2): 995-1005.

Ji, H., Kim, S. R., Kim, Y. H., Kim, H., Eun, M. Y., Jin, I. D., Cha, Y. S., Yun, D. W., Ahn, B. O., and Lee, M. C. 2010. Inactivation of the CTD phosphatase-like gene *OsCPL1* enhances the development of the abscission layer and seed shattering in rice. *Plant J.* 61(1): 96-106.

Keivani, M., Ramezanpour, S. S., Soltanloo, H., Choukan, R., Naghavi, M., and Ranjbar, M . 2010. Genetic diversity assessment of alfalfa (*Medicago sativa* L.) populations using AFLP markers. *Aust. J. Crop Sci.* 4(7): 491-497.

Khush, G. S. 1997. Origin, dispersal, cultivation and variation of rice. *Plant mol. Biol.* 35(2): 25-34.

Kobayashi, A. 1990. Varietal adaptability for mechanized rice cultivation: direct seeding adaptability, shattering habit, smoothness etc. *J. Agric. Sci. Jpn.* 45: 186-189.

Komori, T. and Nitta, N. A. 2004. Simple method to control the seed purity of *japonica* hybrid rice varieties using PCR - based markers. *Plant Breed.* 123(6): 549-553.

Konishi, S., Izawa, T., Lin, S. Y., Ebana, K., Fukuta, Y., Sasaki, T., and Yano, M. 2006. An SNP caused loss of seed shattering during rice domestication. *Science* 312(5778): 1392-1396.

Kumar, V. and Ladha, J. 2011. Direct seeding of rice: Recent developments and future research needs. *Adv. in Agron.* 111: 297-413.

Li, C., Zhou, A., and Sang, T. 2006a. Genetic analysis of rice domestication syndrome with the wild annual species, *Oryza nivara*. *New Phytol.* 170(1): 185-193.

Li, C., Zhou, A., and Sang, T. 2006b. Rice domestication by reducing shattering. *Sci.* 311(5769): 1936-1939.

Liang, H., Deng, Y., Wang, C., and Xu, X. 2015. A high - throughput DNA extraction method from rice seeds. *Biotechnol. Biotechnological Equip.* 30(1): 32-35.

Lin, Z., Griffith, M., Li, X., Zhu, Z., Tan, L., Fu, Y., Zhang, W., Wang, X., Xie, D., and Sun, C. 2007. Origin of seed shattering in rice (*Oryza sativa* L.). *Planta* 226: 11-20.

Londo, J. P., Chiang, Y. C., Hung, K. H., Chiang, T. Y., and Schaal, B. A. 2006. Phylogeography of Asian wild rice, *Oryza rufipogon*, reveals multiple independent domestications of cultivated rice, *Oryza sativa*. *Proc. Natl. Acad. Sci. U.S.A.* 103: 9578-9583.

Mace, E. S., Buhariwalla, K. K., Buhariwalla, H. K., and Crouch, J. H. 2003. High - throughput DNA extraction protocol for tropical molecular breeding programs. *Plant Mol. Biol. Reporter* 21(4): 459-460.

MacRae, E. 2007. Extraction of Plant RNA. *Methods Mol. Biol.* 353: 15-24.

Manak, M. M. 1993. DNA Probes. Keller, G. H. and Manak, M. M. (eds), Stockton Press, New York, pp. 27-68

Mc Couch, S. R., Teytelman, L., Xu, Y., Lbos, K., Clare, K., Walton, M., Fu, B., Maghirang, R., Li, Z., Xing, Y., Zhang, Q., Kono, I., Yano, M., Fjellstrom, R., De Clerck, G., Schneider, D., Cartinhour, S., Ware, D., and Stein, L. 2002. Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). *DNA Res.* 9: 199-207.

Mc Kim, S. M., Stenvik, G. E., Butenko, M. A., Kristiansen, W., Cho, S. K., Hepworth, S. R., Aalen, R. B., and Haughn, G. W. 2008. The BLADE-ON-PETIOLE genes are essential for abscission zone formation in Arabidopsis. *Dev.* 135: 1537-1546.

Mortimer, M., Pandey, S., and Piggin, C. 2000. Weedy rice: approaches to ecological appraisal and implications for research priorities. In: Baki, B. B., Chin, D. V., and Mortimer, A. M. (eds), *Wild and weedy rice in rice ecosystems in Asia - A Review*. Limited proceedings No. 2, International Rice Research Institute, Philippines, pp. 97-105.

Mutou, C., Tanaka, K., and Ishikawa, R. 2014. DNA extraction from rice endosperm (including a protocol for extraction of DNA from ancient seed samples). In: Henry, R. J. and Furtado, A. (eds) *Cereal Genomics. Methods in Molecular Biology (Methods and Protocols), vol. 1099.*, Humana Press, Totowa, New Jersey, pp. 7-15.

Nagai, Y., Sobrizal, S., Sanchez, P. L., Kurakazu, T., Doi, K., and Yoshimura, A. 2002. *Sh3*, a gene for seed shattering, commonly found in wild rices. *Rice Genet. Newsl.* 19: 74-75.

Nagao, S. and Takahashi, M. 1963. Trial construction of twelve linkage group in Japanese rice. *J. Fac. Agric. Hokkaido Univ.* 53: 72-130.

Nam, D. K., Lee, S., Zhou, G., Cao, X., Wang, C., Clark, T., and Wang, S. M. 2002. Oligo (dT) primer generates a high frequency of truncated cDNAs through internal poly (A) priming during reverse transcription. In: *Proceedings of the National Academy of Sciences of the United States of America*, 99(9): 6152-6156.

Olofsdotter, M., Valverde, B. E., and Madesen, K. H. 1999. *Herbicide-resistant rice* (*Oryza sativa* L.) - *A threat or a solution.* Report of the global workshop on red rice control, 30 August - 03 September, Varadero, Cuba, pp. 123-145.

Paterson, A. H. 2002. What has QTL mapping taught us about plant domestication?. *New Phytol.* 154: 591-608.

Pohjanvirta, R., Niittynen, M., Lind´En, J., Boutros, P. C., Moffat, I. D., and Okey, A. B. 2006. Evaluation of various housekeeping genes for their applicability for normalization of mRNA expression in dioxin - treated rats. *Chem.-Biol. Interactions* 160: 134-149.

Powell, W., Machray, G. C., and Provan, J. 1996. Polymorphism revealed by simple sequence repeats. *Trends Plant Sci.* 1: 215-222.

Prathepha, P. 2011. Microsatellite analysis of weedy rice *(Oryza sativa f. spontanea)* from Thailand and Lao PDR. *Aust. J. Crop Sci.* 5(1): 49-54.

Purugganan, M. D. and Fuller, D. Q. 2009. The nature of selection during plant domestication. *Nature* 457: 843-848.

Pyšek, P. and Prach, K. 2003. Research into plant invasions in a crossroads region: History and focus. *Biol. Invasions* 5(4): 337-348.

Qin, Y., Kim, S. M., Zhao, X. H., Jia, B. Y., Lee, H. S., Kim, K. M., Eun, M. Y., Jin, I. D., and Sohn, J. K. 2010. Identification for quantitative trait loci controlling grain shattering in rice. *Genes Genomics* 32(2): 173-180.

Qiu, J., Zhu, J., Fu, F., Ye, C. Y., Wang, W., Mao, L., and Lin, Z. 2014. Genome re - sequencing suggested a weedy rice origin from domesticated *indica* - *japonica* hybridization: a case study from southern China. *Planta* 240(6): 1353-1363.

Rathore, M., Singh, R., and Kumar, B. 2013. Weedy rice: an emerging threat to rice cultivation and options for its management. *Curr. Sci.* 105(8): 1067-1072.

Rathore, M., Singh, R., Kumar, B., and Chauhan, B. S. 2016. Characterization of functional trait diversity among Indian cultivated and weedy rice populations. *Sci. Rep.* 6: 1-9.

Ratnasekera, D. 2015. Weedy rice: A threat to rice production in Sri Lanka. *J. Univ. Ruhuna* 1: 2-13.

Ratnasekera, D., Perera, U. I. P., He, Z., Senanayake, S. G. J. N., Wijesekara, G. A. W., Yang, X., and Lu, B. R. 2014. High level of variation among Sri Lankan weedy rice populations, as estimated by morphological characterization. *Weed Biol. Manag.* 14(1): 68-75.

Reagon, M., Thurber, C. S., Gross, B. L., Olsen, K. M., Jia, Y., and Caicedo, A. L. 2010. Genomic patterns of nucleotide diversity in divergent populations of US weedy rice. *BMC Evol. Biol.* 10:180.

Roy, S. 1921. A preliminary classification of the wild rices of the Central Provinces and Berar. *Agric. J. India* 16: 365-380.

Saitou, N. and Nei, M. 1987. The neighbor - joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4(4): 406-425.

Sang, T. and Ge, S. 2007. The Puzzle of Rice Domestication. *J. Integrative Plant Biol.* 49: 760-768.

Sastry, M. V. S. and Seetharaman, R. 1973. Inheritance of grain shattering and lazy habit and their interrelationship in rice. *Indian J. Gen. Plant. Breed.* 38: 318-321.

Second, G. 1982. Origin of the genic diversity of cultivated rice (*Oryza spp.*): Study of the polymorphism scored at 40 isozyme loci. *Jpn. J. Genet.* 57: 25-57.

Selvey, S., Thompson, E. W., Matthaei, K., Lea, R. A., Irving, M. G., and Griffiths, L. R. 2001. Beta - actin an unsuitable internal control for RT-PCR. *Mol. Cell Probes* 15: 307-311.

Shen, K. A., Meyers, B. C., Islam-Faridi, M. N., Chin, D. B., Stelly, D. M., and Michelmore, R. W. 1998. Resistance gene candidates identified by PCR with degenerate oligo nucleotide primers map to clusters of resistance genes in lettuce. *Mol. Plant-Microbe Interactions*. 11(8): 815-823.

Simons, K. J., Fellers, J. P., Trick, H. N., Zhang, Z. C., Tai, Y. S., Gill, B. S., and Faris, J. D. 2006. Molecular characterization of the major wheat domestication gene Q. *Genet.* 172: 547-555.

Stothard, P. 2000. The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques* 28: 1102-1104.

Subasinghe, S. A. C. C., Ranasinghe, A. M., and Marambe, B. 2007. Molecular characterization of weedy rice in Sri Lanka. In: Marambe, B., Sangakkara, U. R., De Costa, W. A. J. M., and Abeysekara, A. S. K. (eds), *Proceedings of the 21st Asian Pacific Weed Science Society Conference,* 2-6 October 2007, Colombo, Sri Lanka, pp. 469-476.

Subudhi, P. K., Pradeep, K., Singh, T., DeLeon, A. P., Ratna K., Hanamareddy B. M., Cohn, A., and Takuji, S. 2013. "Mapping of seed shattering loci provides insights into origin of weedy rice and rice domestication." *J. Heredity*. 105(2): 276-287.

Suh, H. S., Sato, Y. I., and Morishima, H. 1997. Genetic characterization of weedy rice (*Oryza sativa* L.) based on morpho - physiology, isozymes and RAPD markers. *Theor. Appl. Genet.* 94(4): 316-321.

Sweeney, M. T., Thomson, M. J., Cho, Y. G., Park, Y. J., Williamson. S. H., Bustamante, C. D., and McCouch, S. R. 2007. Global dissemination of a single mutation conferring white pericarp in rice. *PLoS Genet.* 3(8): 1418-1424.

Taghizadeh, M. S., Crawford, S., Nicolas, M. E., and Cousens, R. D. 2009. Water deficit changes the anatomy of the fruit abscission zone in *Raphanus raphanistrum* (Brassicaceae). *Aust. J. Bot*. 57: 708-714.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25(24): 4876-4882.

Thurber, C. S., Jia, M. H., Jia, Y., and Caicedo, A. L. 2013. Similar traits, different genes? Examining convergent evolution in related weedy rice populations. *Mol. Ecol.* 22(3): 685-698.

Thurber, C. S., Reagon, M., Gross, B. L., Olsen, K. M., Jia, Y., and Caicedo, A. L. 2010. Molecular evolution of shattering loci in U. S. weedy rice. *Mol. Ecol.* 19(16): 3271-3284.

Vaughan, L. K., Ottis, B. V., Prazak-Havey, A. M., Bormas, C. A., Sneller, C., and Chandler, J. M. 2001. Is all red rice found in commercial rice really *Oryza sativa*? *Weed Sci.* 49(4): 468-476.

Warude, D., Chavan, P., Joshi, K., and Patwardhan, B. 2003. DNA isolation from fresh, dry plant samples with highly acidic tissue extracts. *Plant Mol. Biol. Reporter.* 21(4): 467a-467f.

Warwick, S. I. and Stewart, C. N. 2005. Crops come from wild plants - how domestication, transgenes, and linkage together shape ferality. In: Gressel, J. (ed.), *Crop ferality and volunteerism*. CRC Press, Boca Raton, Florida, pp. 9-30.

Weising, K., Nybom, H., Wolff, K., and Kahl, G. 2005. *DNA fingerprinting in plants: principles, methods, and applications* (2nd ed.). CRC Press, Boca Raton, Florida, 472p.

Weller, S. C., Bressan, R. A., Goldsbrough, P. B., Fredenburg, T. B., and Hasegawa, P. M. 2001. The effect of genomics on weed management in the 21st century. *Weed Sci.* 49: 282-289.

Wirjahardja, S., Guhardja, E., and Wiroatmodjo, J. 1983. Wild rice and its control. In: *Proceedings of the conference on weed control in Rice,* 31 August-4 September 1981, International Rice Research Institute (IRRI) and the International Weed Science Society (IWSS), Philippines, pp. 319-326.

Xia, H. B., Wang, W., Xia, H., Zhao, W., and Lu, B. R. 2011. Conspecific crop-weed introgression influences evolution of weedy rice (*Oryza sativa f. spontanea*) across a geographical range. *PLOS One* 6(1): 2-11.

Yang, G., Zhou, R., Tang, T., and Shi, S. 2008. Simple and efficient isolation of high - quality total RNA from *Hibiscus tiliaceus*, a mangrove associate and its relatives. *Preparative Biochem. and Biotechnol.* 38(3): 257-264.

Zeigler, R. S. and Barclay, A. 2008. The relevance of rice. *Rice* 1(1): 3-10.

Zhang, L. B., Zhu, Q., Wu, Z. Q., Ross-Ibarra, J., Gaut, B. S., Ge, S., and Sang, T. 2009. Selection on grain shattering genes and rates of rice domestication. *New Phytologist* 184(3): 708-720.

Zhou, Y., Lu, D., Li, C., Luo, J., Zhu, B. F., Zhu, J., Shangguan, Y., Wang, Z., Sang, T., and Zhou, B. 2012. Genetic control of seed shattering in rice by the *APETALA2* transcription factor *SHATTERING ABORTION1*. *Plant Cell.* 24(3): 1034-1048.

Zhu, Q.WW and Ge, S. 2005. Phylogenetic relationships among A - genome species of the genus *Oryza* revealed by intron sequences of four nuclear genes. *New Phytologist* 167(1): 249-265.

Zhu, Y., Ellstrand, N. C., and Lu, B. R. 2012. Sequence polymorphisms in wild, weedy, and cultivated rice suggest seed - shattering locus *sh4* played a minor role in Asian rice domestication. *Ecol. Evol.* 2(9): 2106-2113.

APPENDICES

APPENDICES

APPENDIX I

Dellaporta Extraction Buffer (500 ml, pH= 8.0) :

1*M* Tris (pH 8.0)	100 mM	
0.5 *M* EDTA	50mM	
5*M* NaCl	500mM	
β-Mercaptoethanol	0.2 % (v/v)	Freshly added prior to DNA extraction
PVP	4 % (w/v)	

APPENDIX II

TE buffer (pH= 8.0)

Tris- HCl (pH= 8.0)	10 mM
0.5*M* EDTA	1 mM

APPENDIX III

CTAB DNA Extraction Buffer (500 ml, pH= 8.0) :

C-TAB	2.5 %	
Tris- HCl (pH 8.0)	100 mM	
EDTA	25 mM	
NaCl	1.5 *M*	
β-Mercaptoethanol	0.2 % (v/v)	Freshly added prior to DNA extraction
PVP	4 % (w/v)	

APPENDIX IV

TAE Buffer (50X) for 100 ml solution (pH= 8.2 - 8.5)

Tris base	24.2 g
Glacial acetic acid	5.71 ml
EDTA	1 ml (0.5M)

APPENDIX V

CTAB RNA Extraction Buffer for (500 ml, pH= 8.0) :

C-TAB	2 %	
Tris- Base (pH 8.0)	100 mM	
EDTA	25 mM	
NaCl	2 *M*	
β-Mercaptoethanol	2 % (v/v)	Freshly added prior to DNA extraction
PVP	2 % (w/v)	

APPENDIX VI

GET (Glucose EDTA Tris) buffer (pH= 8.0)

Glucose	50mM
EDTA (0.5 M, pH= 8.0)	10mM
Tris (0.5 M, pH= 8.0)	25 mM

APPENDIX VII

Lysis buffer (pH= 10-12) (0.2 N NaOH/ 1% SDS)

NaOH (1 *N*)	0.2 N
SDS	1%
Distilled water	

APPENDIX VIII

Neutralizing solution (pH= 4.8) (3M potassium acetate)

Potassium acetate (5*M*)	3 *M* (6 ml)
Glacial acetic acid	1.15 ml
Water	2.85 ml

Molecular characterization of shattering in weedy rice (*Oryza sativa* f. *spontanea*) biotypes of Kerala

By

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(2011-09-123)

ABSTRACT

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ABSTRACT

The study entitled "Molecular characterization of shattering in weedy rice (*Oryza sativa* f. *spontanea*) biotypes of Kerala" was conducted at the Integrated Biotechnology Block, College of Agriculture, Vellayani, Thiruvananthapuram during 2015-2017. The objective of the study was isolation and sequencing of genes related to shattering viz. *sh4* and *qsh1* in weedy rice biotypes and characterization by expression profiling and phylogenetic analysis.

Degenerate primers were designed for *sh4* and *qsh1* genes based on the gene sequences retrieved from NCBI database (*sh4* and *qsh1* forward and reverse primers) which were used to isolate and identify the genes. Both genomic DNA and Total RNA of weedy rice and cultivated rice *Uma* were isolated. PCR was done using both genomic DNA and cDNA. Amplification was obtained in two sets of designed primers *i.e. sh4* (FW2 x RW2) and *qsh1* (FW2 x RW2). When DNA was used as template for PCR, two of the *sh4* primers and one of the *qsh1* primers designed for shattering yielded amplicons at the expected size of 649bp, 696bp and 747bp respectively. PCR in cDNA samples of RNA amplicon of size ~690bp with the primer combination of *sh4* (FW2 x RW2) in flag leaf was observed. Also, an amplicon of size ~750bp in flag leaf cDNA was produced using degenerate *qsh1* FW2 x RW2 primers. None of these primers produced an amplification in any of the cDNA samples of cultivated rice *Uma.*

The tBLASTx programme showed that the amplicon of size ~690bp was similar to shattering 4 (*sh4*) genes of *Oryza* populations, whereas the amplicon of size ~750bp belonged to *qsh1* gene for putative transcription factor *qsh1* of rice populations and mRNA of *Oryza sativa japonica* group.

The result clearly revealed that the gene responsible for shattering viz., *sh4* and *qsh1* are present in both weedy rice and cultivated variety *Uma* as evidenced from the PCR amplification of genomic DNA. However, the genes responsible for shattering investigated in the present study viz., *sh4* and *qsh1* were not expressed in any of the growth stages in cultivated rice variety *Uma*.

The NCBI conserved Domain Search programme, showed that the *qsh1* FW2 x RW2 sequence of amplicon at ~750bp (obtained by reaction using weedy rice flag leaf cDNA) belonged to homeobox kn domain. The sequence of the amplicons of *qsh1* obtained was the partial fragments of the first ever *qsh1* gene for putative transcription factor *qsh1* to be isolated from *Oryza sativa* f. *spontanea.*

Phylogenetic tree constructed for *sh4* FW2 x RW2 sequence, had highest similarity to shattering (*sh4*) gene of *Oryza meridionalis* voucher and also had relationship with *Oryza rufipogon* and *Oryza sativa.* However, the weedy rice *qsh1* FW2 x RW2 query sequence was closely related to *qsh1* gene of *Oryza rufipogon,* together with *Oryza sativa indica* group and *Oryza sativa japonica* group. Semi quantitative analysis with *sh4* and *qsh1* gene primers showed that the *sh4* gene was expressed in flag leaf and grains and *qsh1* gene was expressed only in the flag leaf.

From this study it can be inferred that the gens responsible for shattering in weedy rice biotypes of Kerala viz. *sh4* and *qsh1* are present both in cultivated rice and weedy rice. However, the genes are expressed only in weedy rice during the flowering stage.