चावल में पट्टी उत्परिवर्ती का आनुवंशिक विश्लेषण और आण्विक मापन

GENETIC ANALYSIS AND MOLECULAR MAPPING OF *STRIPE* MUTANT IN RICE

मी मी औंग

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आनुवंशिकी संभाग

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GENETIC ANALYSIS AND MOLECULAR MAPPING OF *STRIPE* MUTANT IN RICE

By

Me Me Aung

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CERTIFICATE

This is to certify that the thesis entitled **"Genetic Analysis and Molecular Mapping of *Stripe* Mutant in Rice"** submitted to the Faculty of the Post-Graduate School, ICAR–Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirements for the award of **Master of Science** in **Genetics and Plant Breeding**, embodies the results of bona fide research work carried out by **Ms. Me Me Aung, Roll No. 21052** under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma.

It is further certified that any help or source of information that has been availed of in this connection has been duly acknowledged by her.

Date: 30 July 2019

Place: New Delhi

Dr. Gopala Krishnan S

Chairman, Advisory Committee

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(ME ME AUNG)

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ABBREVIATIONS

µl	Micro liter
bp	Base pairs
BSA	Bulked Segregant Analysis
CG	Completely green
Chl	Chlorophyll
cM	centiMorgan
CTAB	Cetyl- Tri Methyl Ammonium Bromide
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleotide Tri-Phosphates
EDTA	Ethylene Di-amine Tetra Acetate
EMS	Ethyl Methane Sulphonate
MAS	Marker Assisted Selection
mha	Million hectare
MT	Million Ton
Mw	Molecular weight
ng	Nanogram
PCR	Polymerase chain reaction
S	Stripe mutant
SSR	Simple Sequence Repeats
TAE	Tris acetic acid EDTA
Taq polymerase	*Thermus aquaticus* polymerase
TE	Tris EDTA
WT	Wild type

1 INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important staple cereals which plays a pivotal role in food security by accounting for more than 50% of the dietary intake, meeting up to 21% calorific needs of the global population. Out of the total rice production of 495.49 million tonnes across the world, more than 90% of the total production consumed in Asia (FAO, 2019), which accounts for as much as 76% of the calorific requirements of the people residing in South East Asia (Fitzgerald et al., 2009). Rice is grown on 11% of the world's arable land covering an area of 162.50 million hectares, wherein 495.49 million metric tons of production with an average yield of 4.55 tons per hectare. Globally, among the rice growing countries, India has the largest area under rice with ~ 43.79 million hectares (mha). It ranks second behind China with an annual production 112.91 MT (million tons), and 3.87 metric tons per hectare of yield (USDA, 2019; FAOSTAT, 2019). However recently, there has been decline in production of rice, due to shrink natural resources and changing weather patterns, including higher temperatures, severe droughts, floods, salinity and diseases, coupled with more erratic rainfall while the demand for rice is increasing every year. In order to ensure food security for the population in these major rice-consuming countries, there is a need to produce an additional 1.7 MT of rice annually under the limitation imposed by declining area with limited water, labor shortage and fewer chemicals (Salgotra et al., 2018).

Mutation is a very productive breeding tool which has the potential to produce desirable variations in traits that are either not available in nature or that have been lost during domestication. Traditionally, only mutations with economic value in different crops are retained either naturally or with artificial selection, while mutants that are not economically important are generally discarded (Wang et al., 2014). Spontaneous mutations are extremely low in nature and often inadequate in generating genetic variants in traits that are being bred for improving the crops. Various mutagenic agents belonging to physical, chemical and biological have been employed to create mutations that generate genetic variations from which the desirables ones can be selected. Induced rice mutants have been widely used in basic research for identifying the gene(s) affecting different traits as well as in applied

research for developing improved rice varieties. Generally, a mutation breeding is one of the approaches employed to create variants which can help addressing a specific lacuna in an otherwise well-adapted cultivar, which offers the possibility not only directly releasing these mutant as varieties but also as useful donor in breeding programmes (Tai, 2007). A large number of rice cultivars with high yields and superior grain quality have been developed and deployed effectively across the world for improving rice production (Wang et al., 2014). Several induced mutants have been identified in rice which harbors mutations that affect different traits such as plant height, days to flowering, male sterility, grain quality, chlorophyll pigment, abiotic and biotic stress tolerance and yield.

A wide array of mutations affecting leaf colour in rice has been reported, which includes white stripe leaf and zebra leaves. Most the leaf colour mutants reported have been strongly influenced by temperature, wherein the phenotypes of the mutants differed with differences in temperature. Detailed characterization of temperature-sensitiveness of the leaf colour mutants enables us to understand the basis of such leaf colour change, which in turn can help us unravel the relationship between photosynthesis and temperature response. In general, the rice mutants with altered leaf colour have been attributed to the mutations in genes involved in development of chloroplast or metabolism (Zhang and Gao, 2009; Yang et al., 2016; Su et al., 2017). The leaf colour mutants in rice is deficient in chlorophyll content as compared to normal colour wild type, which affects the photosynthetic efficiency and in turn decreases the realized yield (Ye et al., 2017; Ge et al., 2015). In severe instances, such as the albino mutants, the leaves are completely devoid of chlorophyll, which results in plant mortality (Wang et al., 2018). Leaf colour mutants serves as valuable resources for understanding the various leaf morphological, physiological and biochemical processes such as development of chloroplast, its structure and function, chlorophyll metabolism, leaf colour modulation and possibly the resistance to different insect pests and diseases. A number of chlorophyll mutants exhibiting different forms namely, albino, chlorina, stripe, virescent, yellow-green and zebra leaves have been reported in rice. The albino mutant lacks chlorophyll in leaves as a result of which it is completely white in colour and is not able to photosynthesize, which ultimately leads to it mortality soon after germination (Li et al., 2018). The other types of chlorophyll mutants vary in their leaf colour as well as their ability for

photosynthesis based on the nature of mutation. This leads to weak growth in most of the leaf colour mutants, with the extent of weakness depending on the gene/allele governing it. The striped mutant shows white/yellow longitudinal stripes in the leaves, which vary in size as well as the number of stripes. Conversely, the zebra mutant has a typical yellow/white transverse band across the green leaves, which may be variable in its size, frequency and colour. Most of the zebra mutants reported till date has been found to show temporal variation based on the stage of plant growth as well as dependency on the temperature and photoperiod (Kurata et al., 2005).

Mutations are induced in plants through physical, chemical and biological agents. Among these, chemical mutagens such as Ethyl Methane Sulphonate (EMS), offers the advantage of being simple, cheap, and its ability to generate high density point mutations which are random as well as uniformly distributed across the genome (Kim et al., 2004). EMS induced rice mutants have been used to unravel the basic processes involved in tolerance to different abiotic stresses such as stress due to water and salt (Huang et al., 2009; Zhou et al., 2013). These mutants which exhibited better tolerance to salt and heat stress as compared to their wild types have been characterized for their morphological, physiological and proteomic variations (Nakhoda et al., 2012; Poli et al., 2013; Ghaffari et al., 2014; Mithra et al., 2016). A large number of different leaf colour mutants have been developed in rice through induced mutagenesis. In general, these mutant phenotypes show stage specificity observed mainly at seedling stage, and are classified into four different types namely albino, pale green, striped-leaf and spotted leaf based on the differences in their phenotype. Among these, the striped leaf mutants are grouped into two types exhibiting transverse or vertical stripes in the leaves. As described earlier, the mutants showing transverse-stripe in the leaves are called “zebra leaf” mutants, owing to the presence of yellow-green or white-green stripes across the leaves. Till date, 15 different “zebra leaf” rice mutants have been documented in Gramene database, which have been described by different rice researchers across the world. The nature of inheritance in many of these leaf-colour mutants are monogenic recessive, while some of them by dominant or cytoplasmic genes (Qian et al., 1996; Li et al., 2002). The genes governing albino trait in rice are recessive (Iwata et al., 1978), while the yellow-green leaf is monogenic recessive in nature (Li et al., 2010). Many of these genes governing these mutations have been mapped and some of them have been

cloned as well. Zebra mutant rice developed by EMS had lower leaf chlorophyll content and lesser number of chloroplast with aberrant chloroplast structure compared to the wild type (Zhao et al., 2014).

Physical mutagens such as γ-ray irradiation or chemical mutagens such as ethyl methane sulphonate (EMS) have been used to create several rice mutants. Genetic studies involving the characterization of these mutants has helped in mapping genes involved in rice morphogenesis or different traits through linkage mapping and some of them have been cloned as well. As early as 1998, as many as 571 mutant genes were catalogued by the rice genetics committee (Nagato and Yoshimura, 1998). Till date, about 2,000 genes including a large number of quantitative trait loci (QTLs) governing different traits in rice have been documented. While most of the genes show Mendelian segregation, several workers have described the occurrence of non-Mendelian inheritance in striped plants of *Zea*, *Avena*, *Hordeum*, *Oryza* and Sorghum (Robertson, 1937). Additionally, several mutants with significantly higher productivity, better nutrient use/tolerance to deficiency, abiotic stresses or other economically important traits have been developed which when characterized and mapped can be used in rice breeding either through conventional backcross or marker-assisted backcross breeding (Jiang and Ramachandran, 2010) or forward breeding with marker assisted selection (MAS) (Sikora et al., 2011).

A set of 20,000 stable EMS induced rice mutants have been developed as a national resource and are being characterized in rice with the objective of discovering genes governing different traits (Mohapatra et al., 2014; Mithra et al., 2016). Among these stable mutants, a leaf stripe mutant, M507-1 has been identified. The present research was carried out in order to understand the nature of inheritance and map the gene(s) governing stripe leaf mutation in M507-1, with the following objectives:

1. To elucidate the inheritance of *stripe* mutation derived through the EMS induced mutagenesis.
2. Molecular mapping of the *stripe* mutant trait in rice.

2 REVIEW OF LITERATURE

Rice (*Oryza sativa* L.) is major food crop cultivated worldwide and consumed by the half of the world population. The enhancement of rice production and improvement is very important to fulfill the increasing demand for rice especially in the rice consuming population of Asia. A great diversity exists in rice gene pool including the wild relatives of rice which are valuable resources for rice improvement. Rice is cultivated across the globe from tropical regions to northern and temperate regions, despite the limitations imposed by photoperiod and temperature conditions which negatively affect the rice production in many parts of the world (Lu and Chang, 1980). India occupies second rank in area of rice after China with an annual production of more than 104 million tonnes (Babu et al., 2014). In India, rice cultivated in the slopes of the Himalayas from the north-western regions extending to both lowland and upland conditions in many Indian states, Haryana, Punjab, Uttaranchal, Western Uttar Pradesh, Jammu and Kashmir, Himachal Pradesh and Delhi (Salgotra et al., 2015; Wunna et al., 2016).

In the rice breeding programs, the improved germplasms and donors are not adapted and acclimatized to local agro-climatic conditions limiting its use in rice improvement (Juan et al., 2016). Therefore, it is a challenge to plant breeder to incorporate desirable traits in non-adapted varieties which generally possess several undesirable characters and develop varieties with better consumer acceptance (Juan et al., 2016). Development of new high yielding rice cultivars with acceptable quality and tolerance to different stresses is a daunting challenge as most of the traits are complex and are governed by multiple genes. Despite these limitations, various approaches including mutagenesis, transgenics, RNA interference, etc., play an important role in transferring desirable traits aimed at varietal improvement in different of crops namely rice, wheat, maize, chickpea, etc.

Mutation breeding has played an important role in overcoming these limitations through induced mutation either physical or chemical. Mutation breeding has effectively used to improve production in several crops including rice, maize, wheat, and many other crop varieties (Ahloowalia et al., 2004; Riaz and Gul, 2015).

Many induced mutants have been extensively used for genetic studies (rice, maize, sorghum) (Greene et al., 2003). A lot of useful traits have been created which have helped in developing many mutant rice varieties (828 cultivars) which have been documented as Mutant Varieties Database (https://nucleus.iaea.org/Pages/mvd.aspx). The major traits improved include high yield, grain anthocyanin pigmentation, culinary quality, disease resistance, drought tolerance, shorter duration, shorter plant height, and aromatic (Xuan et al., 2019). Several research efforts have been made towards understanding the effect of chemical mutagens on the various characteristics of rice and their role in improving new rice varieties, a brief of which was summarized in the following sections.

2.1 Mutation breeding in rice development

Mutation is an important and an essential evolutionary process to create new variations in plants and animals. While the frequency of spontaneous mutations in plants due to natural factors occur at the very low rate between 10^{-5} and 10^{-8} resulting in adaptation and evolution of crops, it is insufficient for plant breeding, as breeding requires ample genetic variations for the crop improvement with beneficial traits. Therefore, radiation and chemicals mutagens have been used to induce mutations to create new genetic variations, so that preferred mutants can be screened. Mutation breeding has become a very productive breeding tool that allows to induce desired traits that are not available and lost in traditionally used rice varieties during domestication and also for studying functional genomics, breeding, and for understanding the molecular basis of various traits in crops (Wang et al., 2014).

Inducing mutations is relatively a quicker method and a suitable genetic source for creation of genetic variation in crops (Domingo et al., 2007; Ilirjana et al., 2007). Improved rice varieties are one of the best experimental materials in applied generated by mutation breeding in applied research (Tai, 2007). Various mutation studies have been conducted for creating new rice mutants with many important and useful traits, such as yield, plant height, pigmentation, disease/pest resistance, tolerance to abiotic stresses, nutritional qualities, etc., through induced mutants using chemicals and physical mutagens. These have played a significant role in functional genomics study, breeding, and for developing the understanding on molecular mechanism, transcriptomics, proteomics, metabolomics, and phenomics uncovering the molecular bases of biological processes (Morrell et al, 2012; Wang et al., 2014). Alternately, mutations have also been used directly for the development of molecular

maps in structural and functional genomics, which has led to rapid enhancement of yield and quality in crops.

From the past five decades, mutation breeding in agriculture has been used to develop mutant rice varieties through induced mutations using ionizing radiation sources (e.g., X-rays, gamma rays, neutrons) (Micke et al., 1987). Earliest of the rice varieties developed through mutagenesis in rice includes KT 20-74 and SH 30-21, through radiation-induced mutation and released for commercial cultivation in China in 1957, followed by a mutant rice variety, Yenhsing-1, released in Japan (Rutger, 1992). Since then, mutations have been widely used to develop and improve crop varieties for producing desirable variations in important traits such as plant height, flowering time, male sterility, grain quality, abiotic and biotic stress tolerance, and yield (Tai, 2007). The array of induced mutations developed in rice has made it a model species for basic plant research in addition to its key role as the world's most staple crop. Physical mutagens are considered ideal for generating recessive mutations, while chemical mutagens are good for inducing dominant mutant alleles, which may derive new beneficial traits (Shu, 2008). Globally, as many as 443 rice cultivars have been developed through induced mutations using EMS, fast neutron, and gamma irradiation (Kharkwal and Shu, 2009). Chemical mutagens generally produce missense and nonsense mutagenic changes, which alter the functions of genes in the plant genome (Hady et al., 2008). Approximately 100,000 putative mutant rice lines have been generated by mutagens leading to isolation and functional characterization of many genes involved in plant architecture, grain quality and disease resistance (Wang et al., 2014). Several mutant cultivars have been cultivated as improved varieties on a large-scale and had significant economic impact on sustainable crop improvement constantly contribute in food security under in the stress areas of the world (Ahloowalia et al., 2004).

2.2 Role of chemical mutagens in rice improvement

A large number of chemically induced rice mutants have been developed in rice, which have been used in genetic studies (Kurata et al., 2005). Jia et al., (2006) developed a large mutagenized population in the U.S. tropical *japonica* cultivar Katy using ethyl methane sulphonate (EMS), fast neutrons (FN), and gamma irradiation as mutagens. In addition, International Rice Research Institute and their cooperators

have developed a sizable population (about 60,000 mutant lines) of induced mutants in the cultivar IR64 (Wu et al., 2005) using two chemicals namely, EMS and dieoxybutane (DEB), as well as two irradiation sources namely, fast neutrons and gamma rays. These mutants have helped in assigning function to as many as 35,000 genes encoded in the rice genome (International Rice Sequencing Project, 2005). Xuan et al., (2019) produced F_1 generation from the cross TBR1/KD18 using N-methyl-N-nitrosourea (MNU)-induced mutation in rice and generated a mutant population for phenotyping, including stem length, semi-dwarfism, amylose quantity, protein content, gel steadiness, grain yield, and spikelet fertility.

Among the available physical/chemical mutagens, EMS is a simple and cheap chemical mutagen, which generates random irreversible point mutations consistently dispersed in the genome (Kim et al., 2004). This makes it one of the most widely used chemical mutagen to create mutation for developing new variations in crops and it has also been used to create useful breeding materials (Lee et al., 2003). EMS mutants can also be used to understand the functional role of single amino acid residues, creating loss or gain of functions in mutants (Kim et al., 2006). Generally, EMS alkylates of the guanine nucleotide and results in transitions leading to G/C to T/A and A/T to G/C changes with 3-ethyladenine pairing slips at low frequency (Kim et al., 2004). It also creates a large range of mutation in genome with 2 to 10 mutations/Mb in diploid organisms (Till et al., 2007).

EMS is the most potent chemical mutagen which has been widely used not only in rice (Kawai and Sato, 1965; Rao, 1977) but also in other crops for improvement of agronomical and yield attributing traits (Gaul et al., 1972; Jacob, 1965). The EMS induced rice mutants that exhibited more tolerance to drought and salt stress have been used to elucidate the complex mechanism involved in tolerance to water and salt stress (Huang et al., 2009; Zhou et al., 2013a,c). Some EMS induced mutants exhibited better tolerance to heat stress as compared to their wild type (Ghaffari et al., 2014; Poli et al., 2013; Nakhoda et al., 2012; Mithra et al., 2016). An upland drought and heat tolerant rice variety, Nagina 22 (N22) developed before green revolution (Poli et al., 2013; Mohapatra et al., 2014; Pradhan et al., 2016) was used to develop 370 EMS mutants morphologically characterized (Mohapatra et al., 2014; Mithra et al., 2016). More recently, Sevanthi et al., (2018) developed 7,000 more EMS induced mutants in the background of Nagina 22, out of which 541

mutants have been characterized for DUS (Distinctness, Uniformity, and Stability) descriptors as well as chlorophyll and epicuticular wax contents.

2.3 Leaf colour in rice

Leaves in plants are involved in the critical process of photosynthesis, which determines both production as well as productivity. Generally, the mutation frequency in chlorophyll is used an index to evaluate the genetic effects of mutagens as well as to ascertain the efficiency of a mutagenic agent and its dependability (Goyal et al., 2019). Leaf coloration is a function of chlorophyll which may affect the photosynthetic efficacy and grain formation in later stage of rice (Feng et al., 2013). Increasing photosynthesis in leaves is an important feature in breeding cultivars which showed high photosynthetic efficiency (Govaerts et al., 1996; Zhang and Gao, 2009). Leaf-colour mutants are used as visible markers to differentiate plant population developed through mutations for hybrid breeding (Du et al., 2009). The degradation of chlorophyll and breakdown of chlorophyll synthetic pathway such as protochlorophyllide oxidoreductase leads to changes in the leaf-colour in the mutants (Masuda et al., 2003). Rice leaf color mutants has shown decline in photosynthetic efficiency which reduces grain production as well (Ye et al., 2017; Ge et al., 2015) and in severe cases leads to plant mortality (Wang et al., 2018). Leaf color mutants serve as very useful morphological marker to study different physiological and metabolic processes involved in chlorophyll biosynthesis, leaf color variation and variation in chloroplast like structure, function and development (Chen et al., 2018).

A number of leaf color mutants have been reported in several crops including rice (Hu et al., 1981), soybean (Palmer and Rodriguez de Cianzio, 1985), wheat (Marco et al., 1989), maize (Lin and Yu, 1995) and barley (Rudoi and Shcherbakov, 1998). Yellowish-green leaf colour is one of the most common mutant trait observed at seedling stage (Goyal et al., 2019), which tends to revert back to normal green leaf colour at maturity (Thang et al., 2010; Deng et al., 2014). But in some cases, it is constitutive and the leaves remain yellowish green right from seedling to maturity stage (He et al., 2018). For altering leaf colour in rice, chemical mutagens have been used producing several variations including albino, light and altered shaded green, yellow, purple, spotted and zebra stripes (Li et al., 2018) which affects photosynthesis rate, chlorophyll metabolism and chloroplast development. These mutants also play an important role in understanding the mechanism of chlorophyll synthesis and

photosynthesis process for research and practical application in plants. The stripe phenotype observed as a white shaded leaf in the parallel to length of leaf, but different in length size and numbers of white stripes. Another phenotype, zebra has different stripes with white or yellow colours across the green leaves. This phenotype is highly multicolored with variable stripe size, frequency and color at different stage of plant growth (Kurata et al., 2005).

Reddy and Smith, (1981) reported that physical and chemical mutagenic agents can alone or in combination effect mutations affecting the expression of genes involved in leaf coloration in different crop species. The effect of gamma radiation on various phenotypic traits and molecular mechanism on rice seedlings was studied by Hayashi et al., (2014). Alteration of leaf colour at seedling stage into "zebra-type stripes" showing yellow and white colour in entire rice plant was reported by Wang et al., (2009). Temperature sensitiveness on yellow zebra-type stripe mutant has been demonstrated wherein increase in phenotypic expression with increase in temperature (Xie et al., 1995). The white coloured stripes in leaves have been observed after growing the second leaf in seedlings (Xu et al., 2010). Selvan and Raju, (2017) isolated different types of chlorophyll mutant spectrum includes albino, xantha, chlorina and variegated in a traditional rice variety, “Jeeraga Samba” by EMS and colchicine treatment. They also showed that EMS induced higher occurrence and band of chlorophyll mutants than colchicine. Chen et al., (2018) isolated a stable white-lesion mimic leaf mutant induced by EMS in *indica* cultivar, Taichung Native 1 and characterized its chlorophyll content, carotenoids, chloroplast structure and altered expression of genes related with chlorophyll biosynthesis and chloroplast structure in comparison with wild-type. Leaf-color mutant serves as ideal genetic material for unraveling the molecular processes involved in the regulation of chlorophyll biosynthesis and also biodegradation as well as chloroplast structure development.

2.4 Estimation of chlorophyll content

Soil and Plant Analyzer Development meter (SPAD) is an instrument which efficiently measures chlorophyll content in leaf by recording absorption of specific spectrum (Watanabe et al., 1980; Chubachi et al., 1986). It also a new method for correlating nitrogen requirement of crop (Peng et al., 1996; Balasubramanian et al., 2000). SPAD based non-destructive method is being used to measure chlorophyll

content in several crops, such as wheat. The wheat W4 mutant leaves with variegated, albino and green colour leaves at seedling stage showed consistently low value of SPAD units compared to wild wheat (Hamblin et al., 2014). Similarly, Walker et al., (2018) estimated the amount of chlorophyll in mutant soybean leaves using SPAD meter showing yellow, yellow-green, pale green, white colours, which was found to be lower as compared to parental lines. SPAD is easy to handle and use for measuring the chlorophyll content in leaves and also provide accurate data to find out variations in leaf colour.

Dimethyl sulfoxide (DMSO) is another destructive method been used to estimate chlorophyll content in the leaves, because DMSO influences on the mutagenicity which induce by EMS in plant (Khalatkar, 1976). Zhao et al., (2014) observed lower concentration of chlorophyll a, chlorophyll b and carotenoids in zebra leaf mutant (*zb2*) induced by EMS in wild type *japonica* rice cultivar, Nipponbare. Further analysis revealed that the mutation in gene *zb2* resulted in a decline in photosynthetic pigmentation in mutant leaves, especially in yellow coloured regions. In another EMS-induced mutant *sles* derived from *japonica* cultivar, Koshihikari significantly lower amount of chlorophylls and carotenoid was observed as compared to wild type (Kobayashi et al., 2018). In comparison with wild-type, lower chlorophyll content was recorded in EMS induced white striped leaf mutant (*wsl1*), derived from rice cultivar, Nipponbare, during the fourth leaf stage, tillering stage and booting stage (Sheng et al., 2013). These effects of EMS in pigments of leaves suggested that chlorophyll content in rice is reduced due to the deactivation of chlorophyll biosynthesis genes.

2.5 Variation in anatomy of leaves varying in leaf colour

Microtome sectioning of transverse leaves under microscope is used to study the structure of leaves and ultrastructure chloroplast. Mutations in leaves directly associated with chlorophyll and chloroplast result in leaf variegation (Espineda et al., 1999; Gothandam et al., 2005; Zhang et al., 2006; Park et al., 2007; Sugimoto et al., 2007; Wu et al., 2007; Sato et al., 2009), which is observed as different leaf colours namely albino, zebra stripes, chlorina and yellow shaded regions (Yoo et al., 2009). Therefore, mutants with different leaf colors can be considered as ideal genetic resources to study molecular basis of regulation of chlorophyll biosynthesis and

biodegradation as well as development of chloroplast. Transmission Electron Microscopy (TEM) has been used to study chloroplast ultrastructure in freshly harvested leaves from naturally grown five months old plants according to protocol described by Park et al., (2007). Ultrastructure analysis of chloroplasts using TEM revealed that mutants showed two different chloroplast structures with normal thylakoid lamellar structures but without thylakoid lamellar structures (Zhao et al., 2016). EMS induced white stripe mutant rice '*wsl1'* (Gothandam et al., 2005; Sheng et al., 2013) showed degenerated thylakoids and reduced number of chloroplast, lamella and grana compared with wild type. TEM also showed abnormal thylakoid organization with bubbles-like structures and absence of grana lamellae structure in mutant rice (Dong et al., 2001). Zhou et al., (2013b) and Chen et al., (2015) showed decreased levels of chlorophyll in young leaves of mutant pool of *indica* rice compared to their wild counterparts and imaging with TEM displayed loosely arranged thylakoid lamellar in mutant at the maximum tillering stage in contrast to wild type which displayed well-developed lamellar structures. Lee et al., (2018) apparently observed variations in the greenness level of mesophyll cells between wild type and EMS induced mutant of *japonica* cultivar Koshihikari, in dissecting microscope (Olympus, Japan) and the palisade parenchyma part of mesophyll cells in wild plants showed green colour, whereas the spotted regions and mesophyll cells of mutant leaves exhibited completely dark brown indicating the death of leaves. Tan et al., (2019) observed that *ygl14* mutant type F_2 individuals in rice showed the significantly reduced number of chloroplasts and incomplete volume of the chloroplasts in the mesophyll cells in transmission electron micrographs. Moreover, certain chloroplasts had disrupted physical integrity and larger starch granules, whereas others had a normal lamella assembly and starch granules (Sang et al., 2010). These observations indicate that leaf mutations hinder the chloroplast development in the mesophyll cells of rice plants.

2.6 Genes determining leaf colour in rice

About 100 genes have been identified to be encoded by only chloroplast reflecting its semiautonomous nature (Delannoy et al., 2009). The mutation in these genes results in numerous mutants with different vivid visible leaf discoloration phenotypes and also abnormal seedling viability (Zhen et al., 2014; Guo et al., 2015).

Mutations affecting the chloroplast structure, chlorophyll biosynthesis and biodegradation mechanisms cause leaf color alterations (Yoo et al., 2009). In recent years, several genes affecting leaf color have been recognized in monocotyledonous crops such as rice, wheat, maize and sorghum. For example, chlorotic or variegated leaves observed in several discoursed leaf mutants in rice, including *tie-dyed1*, *tie-dyded2*, *camoflauge1*, psychedelic and *sucrose export defective1* (Braun et al., 2006; Baker and Braun, 2007; Ma et al., 2008; Huang et al., 2009; Slewinski and Braun, 2010; Slewinski et al., 2012; Baker et al., 2013). The two *CAO* homologous genes in rice, *OsCAO1* and *OsCAO2*, normally encode an enzyme, chlorophyll a oxygenase, which converts chlorophyll a into chlorophyll b, whereas mutation in gene *OsCAO1* produces different pale-green leaf phenotype compared to normal green colour plant (Lee et al., 2005; Yang et al., 2016).

In rice, seven genes coding for different enzymes magnesium-chelatase ChlD subunit (*OsCHLD*), magnesium chelatase ChlI subunit (*OsCHLI*), magnesium-chelatase ChlH subunit (*OsCHLH*), chlorophyll synthase (*OsCHLG*), divinylreductase gene (*OsDVR*), protochlorophyllide oxidoreductase (*OsPOR*), and chlorophyll a oxygenase (*OsCAO*) were demonstrated to be involved in chlorophyll biosynthesis (Nagata et al., 2004). Non-yellow coloring 1 (*NYC1*) gene encodes chlorophyll b reductase enzyme which inhibits chlorophyll b degradation and produces a stay-green phenotype (Sato et al., 2009). A rice mutant, young leaf chlorosis 1 (*ylc1*) developed from a ^{60}Co-irradiated population showed decreased levels of chlorophyll and lutein due to altered expression of *YLC1* gene which was isolated using map-based cloning (Zhou et al., 2013b). In most of the cases, the mutant genes produce different leaf colours are controlled by a single recessive gene. These can be very useful morphological marker for quickly confirming the purity of hybrid seeds (Zhou et al., 2006).

At least 70 leaf-color mutants identified in rice (Huang et al., 2005; Chen et al., 2007), most of which controlled by recessive nuclear genes such albino genes *nal* (Iwata et al., 1978); yellow green leaf gene, *d83* (Li et al., 2010); thermo insensitive pale green leaf gene, *pgl2* (Zhu et al., 2007); stripe colour gene, *st9(t)*; chlorophyll-deficit *gene, chll2(t)* (Zhang et al., 2010) and temperature-sensitive green-white stripe leaf gene, *gws* (Xu et al., 2010) are recessive nuclear genes, while only a few cases

have been reported, where leaf colour mutant phenotypes are regulated by dominant or cytoplasmic genes (Qian et al., 1996; Li et al., 2012) (Table 2.1). In order to expedite the functional study of mutant genes in rice which regulate leaf colour in chloroplast, many trials have been conducted to develop rice mutant materials using physical and chemical mutagens (Kim et al., 2004; Kolesnik et al., 2004). Therefore, a saturated mutant library can help in reducing the gap between genotypes and phenotypes, and will also useful to identify genetic dissimilarities which are actually valuable for understanding the functions, traits improvement in rice breeding programmes (Thang et al., 2010).

Table 2.1. Genes and its mutagenic source for different leaf colour mutants in rice

S. No.	Leaf colour mutants	Gene symbol	Mutagen	Rice species/ cultivar/ variety	References
1	Stay green Gene	*SGR*	^{60}Co γ-rays at 50 Gy	*japonica* rice cv. Huazhiwu	Jiang et al., 2007
2	Non-yellow coloring	*NYC1*	γ-ray	*japonica* cultivar Nipponbare	Sato et al., 2009
3	Zebra leaf (yellow/green stripes)	*ZEBRA-15*	EMS	*indica* restorer line Jinhui10	Wang wt al., 2009
4	Stripe leaf	*ST1*	Spontaneously induced	*japonica* rice strain FL176	Yoo et al., 2009
5	Virescent leaf	*V3*	Spontaneously induced	Japanese *japonica* Kinmaze	Yoo et al., 2009
6	Virescent colour (white yellow colour)	*wyv1*	EMS	*indica* restorer line Jinhui10	Sang et al., 2010
7	Virescent leaf	*V1*	Low temperature	Taichung 65	Kusumi et al., 2011
8	*Albino*	*Nul1*	EMS	*indica* restorer line Jinhui10	Feng et al., 2012
9	Yellow-green leaf	*ygl2*	^{60}Co irradiation	*indica* Gang 46B	Chen et al., 2013
10	White Striped Leaf	*wsl1*	EMS	*japonica* cultivar Nipponbare	Sheng et al., 2013
11	Young Leaf Chlorosis	*ylc1*	^{60}Co irradiation	*indica* cultivar Zhong 9B	Zhou et al., 2013b
12	Stripe leaf	*ST2*	γ-rays	*japonica* variety, Zhonghua 11(ZH11)	Xu et al., 2014

13	Zebra leaf (yellow/green stripes)	*zb2*	EMS	*japonica* cultivar Nipponbare	Zhao et al., 2014
14	Stripe1-2 and stripe1-3	*st1-2* and *st1-3*	EMS	*indica* 9311	Chen et al., 2015
15	Novel light-dependent lesion mimic	*lmm6*	EMS	*japonica* cv. 02428	Qing et al., 2015
16	Yellow-green leaf	*501ys*	EMS	*japonica* cultivar Nipponbare	Li et al., 2015
17	Leaf lesion mimic mutant 1	*llm1*	EMS	Xa21	Wang et al., 2015
18	Spotted Leaf 3	*spl3*	γ-rays	Japanese *japonica* rice cultivar Norin8	Wang et al., 2015
18	Pale-green leaf mutant	*pgl10*	EMS	*japonica* cultivar Nipponbare	Yang et al., 2016
19	White-stripe leaf	*wsl3*	^{60}Co-irradiated	*indica* rice cultivar 93-11	Zhao et al., 2016
20	Yellow leaf and dwarf 1	*yld1*	EMS	*indica* cultivar Shuhui527	Deng et al., 2017
21	Stripe and drooping leaf	*Sdl*	EMS	*indica* cultivar Zhenong 34	Qin et al., 2017
22	Leaf senescence mutant	*es3(t)*	EMS	*japonica* cultivar Wuyunjing 7 (WYJ7)	Su et al., 2017
23	White stripe leaf4	*wsl4*	^{60}Co-irradiated	*japonica* cultivar RX69	Wang et al., 2017
24	Young Seedling Stripe2	*yss2*	MNU	*japonica* cultivar Nongyuan 238	Zhou et al., 2017
25	Brown Midrib Leaf	*Bml*	EMS	*Indica*cultivar Zhenong 34	Akhter et al., 2018
26	White and lesion mimic leaf	*WLML1*	EMS	*indica* rice cultivar TN1	Chen et al., 2018
27	Rolled Fine Striped	*RFS-1*	γ-rays	*japonica* cultivar Norin-8	Cho et al., 2018
28	Zebra3–1	*z3–1*	N-methyl-N-nitrosourea	*japonica* cultivar Kinmaze	Kim et al., 2018
29	Spotted Leaf Sheath	*sles*	EMS	*japonica* cultivar Koshihikari	Lee et al., 2018
30	Heat-stress sensitive albino 1	*hsa1*	EMS	*japonica* cultivar Nipponbare	Qiu et al., 2018
31	Virescent-albino leaf 1	*val1*	EMS	*indica* restorer line Jinhui10	Zhang et al., 2018
32	Yellow-green leaf 14	*ygl14*	EMS	*indica* restorer line, Xinong 1B	Tan et al., 2019

2.7 Mapping of leaf colour mutants in rice using molecular markers

Leaf colour is widely used as selectable phenotypic and visible marker for testing the genetic purity of seeds in rice (Su et al., 2012). An array of genes for leaf-colour which involved in molecular mechanism and regulation of chlorophyll metabolism in leaf colour rice mutants (Zhou et al., 2012; Sheng et al., 2013). A lot of leaf colour mutant genes have been mapped using molecular markers and out of them, some have been cloned to construct a mutant gene library of rice (Table 2.2). DNA markers have been used to study genetic diversity, functional genomics and structural characteristics of genomic DNA. Among them, Simple Sequence Repeats (SSRs) are widely applied due to their highly reproducible, co-dominant nature, sequence specific and highly polymorphic and abundantly dispersed throughout the rice genome (Akagi et al., 1996; McCouch et al., 1997; Wu and Tanksley, 1993). SSRs comprise of tandem repeats of short DNA motifs (1-6 bp in length) that exhibit higher genetic variation due to differences in the number of repeats occur at a locus (Litt and Luty, 1989). Molecular markers like SSRs are neutral to environmental factors and growth practices, whereas morphological and biochemical markers frequently affected by these constrains (Ovesna et al., 2002). SSR markers have been used for the identification and mapping of gene/QTLs in plants and animals for a long time. A large number of SSRs have been developed and mapped in rice showing a great degree of polymorphism for their position in genome, numbers of repeat motifs and the genome wide distribution (Temnykh et al.,2000; McCouch et al., 2002).

Molecular breeding in rice has provided the advantage of identifying QTLs linked to many desired agronomic and physiological traits which are controlled by more than one gene and these genes show complex and quantitative inheritance (Takai et al., 2010). As QTLs with agronomic assessment have been labelled using DNA markers and transferred into promising genotypes/cultivars through marker-assisted selection (MAS) (Fukuoka et al., 2010). Now, the complete sequence of the rice genome is available and very useful to facilitate the genetic study of the desired traits (IRGSP, 2005). From the genome sequence, a large number of SSRs have been developed, which are the main sources of genetic markers and conveniently and effectively used in breeding to develop new rice cultivars with important agronomic traits such as stem length, number of seeds, flowering time, maturity, panicle length,

seed shattering, chlorophyll content, amylose content, yield and resistance to disease and abiotic stresses (Xie et al., 2014).

In rice, SSRs have been extensively used to map leaf colour mutant genes namely, *OsCHLH9* (Goh et al., 2004), *Chlorina-1, Chlorina-9* (Zhang et al., 2006), *OsDVR* (Wang et al., 2010), *virescent1* and *virescent2* (Hiroki et al., 2004) located on chromosome 3; *YGL1* (Wu et al., 2007), *OsHAP3B* and *OsHAP3C* (Kazumaru et al., 2003) delimited on chromosome 5; *NYC3* (Ryouhei et al., 2009), *virescent3* (Yoo et al., 2009) and *SPP* (Yue et al., 2010) mapped on chromosome 6; *OsPPR1*, *sgr* (Kodiveri et al., 2005) and *cisc(t)* (Lan et al., 2007) delimited on chromosome 9.

Using newly developed insertion/deletion known as InDel markers and SSRs, Chen et al., (2018) mapped a recessive nuclear gene (*WLML1*) with a distance of 159.7 kb region from gene on chromosome 4 after developing a leaf colour mutant white and lesion mimic leaf1 (*wlml1*) induced by EMS from the *indica* cultivar Taichung Native 1. Another rice leaf colour controlling single recessive gene (*wsl1*) have also been identified in white striped leaf mutant (*wsl1*) using an F_2 population derived from *wsl1*/ Nanjing 11 and mapped on the telomere region of chromosome 9, positioned between RM23742 and RM23759 with approximately 486.5 kb (Sheng et al., 2013). A set of recessive plants from the F_2 population derived from the cross between *ys83*/ Minghui63 was used for fine mapping mutant gene in yellow-green leaf in rice using SSR markers (McCouch et al., 2002). Bulked Segregant Analysis (BSA) was applied to determine the approximate map position of *WSL12* in the F_2 individuals of the cross wsl12/93-11 showed mutant leaf colour and *WSL12* was located on chromosome 12 close to RM1246 (Ye et al., 2016). Polymorphic SSRs used for primary mapping a gene of leaf mutant phenotype in the F_2 population through BSA located the mutant gene on chromosome 3 with the genetic distances of 86.0 cM and 91.1 cM, respectively, between SSR markers CHL-8 and RM16 (He et al., 2011).

A stripe leaf mutant gene, *st(t)*, identified in the mutant line developed from *indica* restorer Jinhui 10 induced by EMS and mapped it on chromosome 6 between two SSRs RM19745 and RM19762 flanked with genetic distances of 0.07 cM and 0.27 cM, respectively. Map-based cloning was used to map a young leaf chlorosis 1

(*YLC1*) locus at genetic distance of 8.9 cM interval chromosome 9L between SSR markers RM105 and RM5657 using ^{60}Co-irradiation in F_2 population from the 02428/*ylc1* in leaf tissues of rice (Zhou et al., 2013b). A yellow green leaf mutant (*ygl14*) derived from *indica* restorer line 'Xinong 1B' of rice treated with EMS used to fine map gene *YGL14* located between two SSR markers, ZTQ48 and RM3664 with genetic distance of 23.86 cM and 21.59 cM, respectively, in certain region of 70.7 kb on chromosome 5 (Tan et al., 2019).

In a first sequencing-based approach, as many as 900–2,000 EMS induced mutations in rice were used to map zebra stripe mutant (Abe et al., 2012). Additionally, F_2 population in the background of Nagina 22 treated with EMS was used to map as many as 81 genes across the whole genome of rice using SSRs, SNPs and InDel markers (Sevanthi et al., 2018). A *Zebra-15* gene was tagged on chromosome 5 at a distance of 19.6 cM from RM3322 as well as 6.0 cM from RM6082 from the 150 F_2 recessive individuals derived from the *indica* rice restorer line Jinhui10 treated by EMS (Wang et al., 2009). Another zebra mutant gene *zl2* was also mapped with the interval of about 164.3 kb on chromosome 11 in a "zebra leaf" mutant (*ZEBRA LEAF 2*) obtained from tissue culture and induced by EMS (Liu et al., 2013). Similarly, from a EMS induced mutant of a *japonica* rice cultivar, 'Nipponbare' a single recessive nuclear gene, *ZB2* was mapped using 18 SSRs markers and positioned onto 111.9 kb in chromosome 9 using map-based cloning (Zhao et al., 2014). The review of literature on leaf colour mutation especially in rice shows that induced mutations are powerful and efficient strategy to map the genes and functional characterization of the genes governing leaf colour, development of chloroplast structure and pigment production in rice leaves. Further in-depth studies can be helpful in developing an understanding on the role of these genes in chlorophyll synthesis and it subsequent effect on photosynthetic efficiency in rice.

Table 2.2. Genes and linked markers identified for different leaf colour mutants in rice

Leaf colour/traits	Gene	Chr. No.	Marker Type	Marker name	Distance from marker	Reference
Chlorophyll deficient 1	*cde1(t)*	2	SSR	RM7082, RM7636	25.5 cM, 36.8 cM	Liu et al., 2007
Chlorophyll deficient	*cde1(t)*	12	SSR	RM 6938, RM 7562	4.7 cM, 8.6 cM	Liu et al., 2007
White stripe	*st1*	6	SSR	RM5855, RM3431	9.8 cM	Yoo et al., 2009
Virescent3	*v3*	6	SSR	RM204, RM6734	4.7 cM	
White stripe leaf	*st10*	3	SSR	STR19, STR24	0.15 Mb	He et al., 2011
Green-white-green leaf	*GWGL*	12	InDel	L59.2-7, L64.8-11	0.74 Mb	Li et al., 2012
Low temperature albino 1	*lta1*	11	SNP and InDel	SNP21, InD43	0.11 Mb	Peng et al., 2012
Yellow-green leaf	*ygl2*	6	InDels	In 44, In 39	0.66 Mb	Chen et al., 2013
Novel white striped leaf	*wsl1*	9	SSR	RM23742, RM23759	0.49 Mb	Sheng et al., 2013
Spontaneous yellow-green leaf	*ygl7*	3	SSR and SFP	RM1308, SFP-3-6	3.9 cM	Deng et al., 2014
Stripe 2	*st2*	1	SSR and CAPS	RM8139, C7962	6.5 cM	Xu et al., 2014
Spotted leaf	*spl30*	9	SSR	RM6543, RM7697	8.5 cM, 0.6 cM	Xu et al., 2014
stripe1-2	*st1-2*	6	SSR	RM3438, RM3431	3.3 cM, 2.2 cM	Chen et al., 2015
Novel Vegetative Senescence Lethal	*Vsl1*	8	SSR	RM22720, RM22926	0.47 cM, 0.57 cM	Yin et al., 2015

Pale-green leaf mutant	*pgl10*	10	SSR	RM258, RM3019	5.1 cM, 3.6 cM	Yang et al., 2016
White stripe leaf	*WSL12*	12	SSR	RM1246	0.03 Mb	Ye et al., 2016
White-stripe leaf	*wsl3*	1	SSR	RM323, RM3740	0.51 Mb	Zhao et al., 2016
Yellow-green leaf	*YGL8*	1	SSR and InDel	RM6141, RM6321	0.05 Mb	Zhu et al., 2016
yellow leaf and dwarf 1	*yld1*	6	SSR	RM345	7.3 Mb	Deng et al., 2017
light-green leaves	*lgl1*	12	InDel	RM101, RM1246	48.2 cM, 65.3 cM	Mei et al., 2017
White stripe leaf	*WSL4*	2	InDel	L-37, L-26	0.08 Mb	Wang et al., 2017
Yellow-green leaf	*ygl1*	5	CAPS	P23, P8	0.01 Mb	Wu et al., 2017
Young Seedling Stripe2	*yss2*	12	Indel/ SSR	ID12-8, RM3331	5.4 Mb	Zhou et al., 2017
White and lesion mimic leaf	*WLML1*	4	InDel	P15, P16	0.16 Mb	Chen et al., 2018
zebra3	*z3*	3	STS and SSR	STS1, RM7576	3.91Mb	Kim et al., 2018
White sterile line No.01	*W01S*	3	SSR	RM411, RM2346	3.40 cM, 4.56 cM	Li et al., 2018
Yellow-green leaf 14	*ygl14*	5	SSR and InDel	ZTQ48, RM3664 (SSR), T9, T10 (InDel)	23.86 cM, 21.59 cM, (SSR), 0.71 Mb (InDel)	Tan et al., 2019

3 MATERIALS AND METHODS

Food security of the world is primarily dependent on rice (*Oryza sativa* L.) production, as the diet of more than 50% of the global human population is based on the rice staple. The demand for rice is increasing and is expected to increase further as the population is rising especially in Asian countries and the limitations for growing rice is also increasing with constraints imposed by climate change, environmental stress, and pest and disease infestation. Induced mutation is a very potential tool, which offers the possibility of creating trait variations that may be desirable, which are not found in nature or that have been lost during domestication. Mutations causing change in leaf colour in rice namely, such as the white stripes, yellow stripes and zebra pattern have been reported, some of which are heavily influenced by growth conditions such as temperature and photoperiod. As the change in leaf colour is caused by changes in the chloroplast structure and number, it is expected to have potential impact on the photosynthetic efficiency, which in turn will also affect rice productivity. Therefore, research on leaf colour mutation is of paramount importance in understanding the basis of leaf colour change and its effect on photosynthetic efficiency.

The present investigation was carried out in the rice mutant, M507-1, developed through EMS induced mutation of a popular drought and heat tolerant upland rice cultivar Nagina 22. The mutant, M507-1, exhibits a typical white striped pattern in leaf blade, leaf sheath and the glumes. The present chapter describes the genetic materials used in the study for unraveling the nature of inheritance and molecular mapping of the gene(s) governing stripe leaf trait in the mutant, along with the techniques and methods used during the course of investigation.

3.1 Genetic materials

In order to characterize the inheritance pattern of the stripe leaf mutant, the genetic materials including the white stripe leaf rice mutant ‘M507-1’ derived from an upland rice cultivar, Nagina 22 through EMS induced mutation, its wild type parent, Nagina 22 and Pusa 44 were used. From these genotypes, two different

mapping populations were developed by crossing the white stripe leaf mutant, 'M507-1', with normal green coloured leaf genotypes including the wild type, Nagina 22 and Pusa 44, separately, during *Kharif* 2016 at the experimental farm of Division of Genetics, ICAR-Indian Agricultural Research Institute (ICAR-IARI), New Delhi. The F_1 seeds from both the crosses were raised at the offseason nursery, Rice Breeding and Genetics Research Centre (RBGRC), ICAR-Indian Agricultural Research Institute (ICAR-IARI), Aduthurai and selfed to generate F_2 populations.

The F_2 populations from both these crosses (M507-1/Pusa 44 and M507-1/Nagina 22) along with the parental lines were raised at Division of Genetics, ICAR-IARI, New Delhi during *Kharif* 2017 for phenotypic characterization of leaf colour and molecular analysis. Twenty-one day old single seedlings were transplanted at a spacing of 20 cm x 15 cm and phenotypic observations on leaf colour of each of the single plants were recorded. Simultaneously, the leaf samples from each of the individual plants were collected for genotyping in both the F_2 populations.

3.1.1 Estimation of photosynthetic pigments of the parents

Two methods used to estimate the content of pigments in stripe leaf mutant (M507-1) and the normal green colour genotypes, Pusa 44 and Nagina 22. The first method involved the use of SPAD chlorophyll meter to determine chlorophyll concentration, while the other method was spectrophotometric assay based on the protocol described by Arnon (1949) using DiMethyl SulfOxide (DMSO) method to estimate photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid contents) in the leaves of the genotypes at four different stage namely seedling, tillering, booting and heading stages. Fresh leaf tissues (50 mg) were collected and soaked in 5 ml DMSO, sequentially extracted pigments after keeping leaves for overnight at room temperature. Then residual plant debris was removed by centrifugation at 10,000 rpm for 10 minutes and the supernatants transferred into fresh tubes. Three biological replicates were prepared for each plant to analyze with a microprocessor UV-Vis double beam spectrophotometer model No-HB-2007. The optical density of the sample solutions measured at three different wavelengths 663, 645 and 470 nm. The contents of chlorophylls (Chl a, Chl b and total chlorophyll) calculated in leaves of mutant and wild plants according to Arnon's equation (1949).

$$\text{Chl a} = \frac{(12.7 \times Abs_{663}) - (2.6 \times Abs_{645}) \times 10 \text{ ml of acetone}}{100 \text{ mg leaf tissue}}$$

$$\text{Chl b} = \frac{(22.9 \times Abs_{645}) - (4.68 \times Abs_{663}) \times 10 \text{ ml of acetone}}{100 \text{ mg leaf tissue}}$$

$$\text{Chl a+b} = \frac{(22.9 \times Abs_{645}) - (4.68 \times Abs_{663}) \times 10 \text{ ml of acetone}}{100 \text{ mg leaf tissue}}$$

Total carotenoid content was also determined by measuring the absorbance at 470 nm and calculated according to the equation given by Lichtenthaler (1987) which was expressed as milligrams of carotenoid per gram of plant tissue based on the following formula:

$$\text{Carotenoids (x+c)} = \frac{1000 \times Abs_{470} - 1.82 \times Chl_a - 85.02\ Chl_b}{198}$$

Where,

Chl a = Concentration of Chl a in micrograms per ml of plant extract.

Chl b = Concentration of Chl b in micrograms per ml of plant extract.

x + c = Xanthophylls and carotenes

A_{470}, A_{645} and A_{663} = Absorbance values at 470, 645 and 663 nm, respectively

3.1.2 Phenotypic Characterization of the Mutant

The mutant genotype, M507-1, exhibited alternate stripes of white and green leaves from seedling to maturity stage while the plants of wild type, Nagina 22 and Pusa 44 possesses complete green leaves (Figure 1). Each of the plants from F_2 populations was phenotyped for leaf colour trait as striped with white-green stripe and complete green leaves, visually. Leaf colour was scored as CG for plants producing

complete green leaves (wild type) while the plants showing stripe mutant type leaf colour with alternate white and green stripes were scored as S.

3.1.2.1 Statistical data analysis

The observed phenotypic data of the F_2 and the phenotypic and genotypic data a subset of the F_2 population used in mapping were analysed for the goodness of fit with the expected non-Mendelian segregation ratio using chi-square (χ^2) test using the following formula:

$$\chi^2_{(n-1)dt} = \sum_{i=1}^{k} \frac{(O_i - E_i)^2}{E_i}$$

Where, O_i is the observed data and E_i is the expected data. The test for goodness of fit was carried out with the Null hypothesis (H_0) that there is no difference between expected and observed frequency. The Null hypothesis was accepted, if the p-value was more than 0.05 for (n-1) degree of freedom (d_f). Whenever, the calculated value p-value was less than 0.05, the null hypothesis was rejected and fitness to alternative ration was tested.

3.1.3 Histological analysis of the rice leaves

3.1.3.1 Fixation in FAA, Embedding, Sectioning and Feulgen Staining

Young developing rice leaves of the stripe mutant, M507-1, Pusa 44 and Nagina 22 were processed for microscope analysis of tissue sections (Kladnik, 2013). The tissues were collected and preserved in the fixative FAA (formaldehyde, acetic acid and ethanol) till further processing of the samples. The plant materials, reagents and equipments used for fixation, sectioning and staining the tissues is presented in Appendix I.

Fixation in FAA

- 10 ml aliquot of the FAA fixative was dispensed in ~ 20 ml glass vials kept on ice, so as to keep the fixative chilled. The leaf tissue was cut into pieces of less than 2 or 3 mm with a scalpel or razor blade, and immediately immersed in cold fixative. The size of the pieces of the tissue was sufficient for the section that was to be made for further analysis.

- The open vials with fixed material was then placed in a vacuum desiccator and exposed to moderate vacuum for 15 minutes so as to remove the air out of the tissue. The tissue starts sinking when the vacuum is released, if not then this step was repeated once again to release the vacuum, after which fresh fixative was added in vials and the tissues were allowed to get fixed overnight at 4°C.

Dehydration in a series of Tert-butanol (TBA)

- The pure TBA (1L) is placed on the top of an incubation oven at 56-60°C to warm it before use, as it tends to freeze below 25°C. The dehydration solutions were prepared and the solutions a–e (Appendix I) were stored at room temperature (RT) while the solutions (Appendix I) on the incubating oven at 56-60°C to keep it warm.

- The tissue was dehydrated in each step and kept for one day, under a fume hood. The old solution is taken out from the vial using a plastic Pasteur pipette and replaced with equal amount of next solution. At the last step, TBA is filled upto half the volume of the vial so as to leave enough space for adding Paraplast Plus in the following step.

Embedding in paraffin (Paraplast Plus)

- A large stock of approximately 300 ml of molten Paraplast Plus in two rectangular histology staining dishes and the plastic pipettes are incubated in an oven at 56-60°C.

- The pellets of Paraplast Plus wax were added in vials half filled with TBA. The fine structure Paraplast Plus and additives (DMSO) enable it to penetrate effectively into the tissue, which helps in obtaining good sections. The vials were capped and incubated at 56-60°C in an oven with intermittent mixing, till the wax was completely melted and incubated in the oven, overnight.

- The next day, TBA/wax mixture is decanted into a waste container made of aluminium foil under a fume hood. Fresh molten wax is added and incubated in oven, overnight with vials open, so that all the TBA evaporates. For the next two days the fresh molten wax is again added several times after decanting the old wax for two times a day.

3.1.3.2 Casting wax blocks

First of all, the lab in which the casting is kept warm by adjusting the air condition to warm temperature as colder temperature will solidify the wax too quickly. A small amount of molten wax was kept in a beaker in the aluminium foil tent in the hot plate. The hot plate is protected with a layer of aluminium foil to keep it free from being smudged with wax. The temperature of the hot plate is checked from time to time so that the temperature is regulated in such a way that the wax does not solidify while the hot plate also does not get too hot to touch and prolonged temperatures over 62°C will also damage Paraplast Plus. The metal molds are also placed on the hot plate before using them.

One vial with samples was taken out of the oven at a time, and placed on the hot plate. The embedding rings were properly labelled with a pencil or water proof marker with the sample number. Some wax was poured in the mold and one piece of leaf tissue was taken of the vial with forceps warmed in a flame of alcohol burner. Care was taken that the forceps were too hot.

The sample was placed on an orientation in the mold on the hotplate, so as to obtain the desired sections. The mold was then carefully transferred to a brass-plate/ice-packet or just on the table, so that the bottom layer of wax solidified and fixed the sample in place. Care was taken if the sample was not positioned in a desired manner on the hot plate; it was positioned, when the bottom layer of wax started solidifying. The mold with molten wax filled to the edge, and covered with the labeled embedding ring were then transferred into a tray with cold water and ice and left for about 10 minutes, so as to allow the wax to get solidified completely. Then the mold was put on the ice, waited for a few minutes and removed the wax block from the mold (the cooling with ice helped the block getting loose from the mold). The mold was placed back on the hot plate for re-use. At a time, about 5 to 10 blocks were processed, which were then stored in the refrigerator at 4°C still further processing.

3.1.3.3 Sectioning

The wax blocks were trimmed around the sample with a single-edged razor blade, so that the upper and lower edges were parallel, and the left and right edges were at an angle, so that the lower edge of the block was longer than the upper. Also

the sides of the block were shaped with the razor blade in such way that it inclined away. Care was taken that the razor blade were sharp enough to shape the blocks with the sample in clean cuts from the top to the bottom of the block.

The embedding rings with the sample were then fixed in the sample holder on the microtome. The thickness of sections was set to 10 - 20 µm with a cutting angle of around 7 degrees. Once the microtome, starts sectioning, the first few sections were usually not good and therefore, they were removed with a small brush, away from the blade. When the surface of the sample was even, the ribbon started forming and enough sections were cut to fit them on a dark cardboard, and the ribbon was cut with a razor blade. Several ribbons were cut, until the region of interest was cut. The sections were viewed with a stereomicroscope, to locate the sections of interest (e.g. median longitudinal sections of the kernel).

The objective slides were labelled with a pencil and placed on the hot plate maintained at 40°C and some water was applied to the slides such that it almost covered the slides. The ribbon was cut in smaller regions with two or three sections, and two sets of sections were applied on each slide, so that one was used for the experiment and the other served as a control. It was ensured that the water area was larger than the size of the ribbon, so that the sections have room to spread and even out. Any excess water from the slides was drained using filter paper. The sections were left on the hot plate overnight to dry and attach to the slides after which they were stored in slide boxes at room temperature or in the refrigerator at 4°C till further processing.

3.1.3.4 Feulgen staining

The Feulgen reaction quantitatively stains the DNA (Feulgen and Rossen beck, 1924), which makes the nuclei is stained purple, while the rest of the cell remains clear. The staining was done using 40 ml staining solution in Coplin jars. Sections on objective slides were dewaxed in xylene, rehydrated through an ethanol series to water, hydrolyzed in 5 M HCl at 20°C for 75 minutes, stained with Feulgen reagent at 20°C for 120 minutes, washed for 45 minutes in six changed of SO_2-water, dehydrated in an ethanol series, then mounted in DPX mounting medium.

Table 3.1. Feulgen staining protocol (adapted from Greilhuber and Ebert, 1994)

Rehydration	100% xylene 100% xylene Absolute alcohol 96% alcohol 70% alcohol 30% alcohol Distilled water	RT 5 min/step	Date of solution preparation Feulgen: HCL: SO_2 water: Xylene: Ethanols:
Hydrolysis	5M HCl (in a water bath) Distilled water (cold water stops hydrolysis)	20°C -----min 4°C 5 min	75 min for FAA fixed Paraplast embedded samples
Feulgen staining	Feulgen (water bath)	20°C 120 min	
Washing	SO_2 water (work in fume hood)	RT 3 x 2 min 2 x 10 min 20 min	250 ml SO_2 water 247.5 ml dH_2O 2.5 ml 5M HCl 1.25 g $K_2S_2O_2$
Dehydration	Distilled water 30% alcohol 70% alcohol 96% alcohol absolute alcohol 100% xylene 100% xylene	RT 5 min/step	
Mounting	DPX + cover slip		

3.2 Molecular mapping

Two mapping populations from the crosses, M507-1/Pusa 44 and M507-1/Nagina 22, were used for mapping the stripe leaf mutant gene using F_2 population. Young leaf samples were collected from each plant of the F_2 population as well as the parents, individually at maximum tillering stage for extraction of the genomic DNA.

3.2.1 Methodology

3.2.1.1 Isolation of DNA

For the extraction of genomic DNA, the chemical reagents such as Cetyl-trimethyl ammonium bromide (CTAB), Trizma-base, Ethylene Diamine Tetra Aacetate (EDTA), Sodium chloride, Chloroform: Isoamyl alcohol mixture, RNase and Ethanol were prepared with molecular biology and analytical grade (Appendix IV). The DNA extraction buffer (DEB) was prepared as per the standard protocol (Appendix V).

The extraction of genomic DNA was done using the procedure described by Maroof et al., (1984) with some minor modifications:

1. The leaf samples (2 g) were weighed and homogenized in 2 ml CTAB buffer (preheated to 65°C) using leaf grinding tissue lyser (Qiagen).
2. The fine paste was then transferred to a 2 ml centrifuge tube and mixed thoroughly.
3. The samples were incubated in a water bath at 65°C for 1 hour with gentle shaking after every 15 minutes.
4. After incubation, the samples were taken out from the water bath and allowed to cool down at room temperature.
5. An equal volume (to DEB) of the mixture of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed thoroughly but gently for not less than 5 minutes.
6. The mixture was then centrifuged for about 10 minutes at 10,000 rpm at room temperature.
7. Supernatant was obtained and then transferred to a 1.5 ml fresh tube.
8. An equal volume (to supernatant) of pre-chilled isopropanol was added and mixed gently by inverting the tubes and kept overnight at -20°C.
9. The DNA precipitate was then spooled out and transferred to a 1.5 ml micro-centrifuge tube.
10. DNA was again pelleted by centrifugation at 10,000 rpm for 10 minutes.
11. The supernatant was now discarded and pellet was washed twice with 70% ethanol by centrifugation for 5 minutes at 10000 rpm.

12. The pellet was dried up at room temperature and dissolved in 100 µl of $T_{10}E_1$ (pH 8.0) buffer with RNAase (10 mg/ml) and stored at -20°C for further use.

3.2.1.2 Quantification of DNA

Quality and quantity of DNA were determined by UV/Vis nano spectrophotometer (Microdigital Co. Ltd., Gyeonggi-do, Republic of Korea). Purity of DNA was checked by taking the ratio of optical density (OD) using spectrophotometer, at 260 nm to that of 280 nm. The samples with OD ratio (260 nm/280 nm) between 1.7-1.9 were used in subsequent experiments. DNA samples showing the values beyond this range were re-purified.

3.2.1.3 Dilution of DNA

Depending on the concentration of DNA, samples were diluted with accordance amount of $T_{10}E_1$ buffer and again checked through UV/Vis nano spectrophotometer (Microdigital Co. Ltd., Gyeonggi-do, Republic of Korea) to acquire final dilution. The quantified DNA was diluted with in $T_{10}E_1$ according to the required DNA concentration in each sample (25ng/µl) for further use in PCR amplification.

3.2.1.4 Survey of parental polymorphism

A total of 1114 markers uniformly distributed across all twelve rice chromosomes were used for survey of parental polymorphism between parents (M507-1/Pusa 44) and further analysis. Simple sequence repeat markers (SSRs) were obtained from the Gramene Database (http://www.Gramene.org) and a set of hyper variable SSR (HvSSR) markers containing di-, tri- and tetra-nucleotide repeat motifs from the 12 rice chromosomes (Singh, 2009). The details of polymorphic markers used along with the information of physical positions, primer sequences are presented in Appendix VI.

3.2.1.5 PCR amplification with SSR markers

Polymerase chain reaction (PCR) reaction of 10 µl volume was set up using 20-30 ng template DNA, 5 pmol of each primer, 0.05 mM dNTPs (MBI, Lithuania, USA), 10x PCR buffer (10 mM Tris, pH 8.4, 1.8 mM $MgCl_2$) and 0.5 U of *Taq* DNA polymerase (Banglore Genei Pvt. Ltd., India).

Table 3.2. Reaction mixture for PCR to detect SSR markers

Reagents	Stock concentration	Aliquot volume	Final Concentration
DNA	25 ng/µl	1.0 µl	25 ng
PCR buffer	10 x	1.0 µl	1x
dNTPs	2 mM	1.0 µl	0.2 mM
Forward primer	5 pM	0.5 µl	2.5 pM
Reverse primer	5 pM	0.5 µl	2.5 pM
Taq Polymerase	3 unit/µl	0.2 µl	0.6 U
Milli-Q water	-	5.8 µl	-
Total	-	10.0 µl	-

PCR was performed in a thermal cycler (Agilent, USA) with the following thermal regimes mentioned in Table 3.3.

Table 3.3. Temperature profile used in PCR for SSR amplification

Steps	Temperature	Duration	Cycles	Activity
1.	95°C	5 min	1	Initial denaturation
2.	95°C	30 sec	35	Denaturation
3.	55°C	30 sec		Annealing
4.	72°C	30 sec		Elongation
5.	72°C	10 min	1	Final elongation
6.	4°C	∞		Storage

3.2.1.6 Bulked Segregant Analysis (BSA)

To identify the marker putatively linked to stripe leaf mutant trait, Bulked Segregant Analysis (BSA) proposed by Michelmore et al., 1991 was carried out. DNA isolated from 10 fully complete green (wild type) and white stripe (mutant type) plants were used to constitute the wild bulks and mutant bulks, respectively. BSA with the parental lines, 'Pusa 44' and 'M507-1' along with wild and mutant F_2 bulks was carried out with markers polymorphic between the parents. Based on inheritance pattern observed, a modified strategy of two steps Bulked Segregant Analysis, BSA1

and BSA2, were carried out to map the two gene(s) governing stripe leaf mutation. The putatively linked markers were then used for genotyping of the individual F_2 plants for linkage analysis.

3.2.1.7 Resolution of amplified PCR products

The PCR products were resolved using 3.5% agarose gel, wherein 17.5 g of agarose powder was suspended in 500 ml of 1x TAE buffer and heated in microwave oven till clear solution was obtained. The solution was allowed to cool down to room temperature and 25 µl (0.05 µl/ml of 1x TAE) of ethidium bromide (EtBr) stock solution (10 mg/ml of double distilled water) was added. The gel was gently stirred and poured into the gel casting tray. The combs were approximately placed to produce wells for loading PCR products. After 30-45 minutes when proper solidification occurs, the gel was shifted to the electrophoresis tank filled with 1x TAE buffer (pH 8.0) and the combs were removed carefully from the gel without disturbing the wells. To each PCR product of 10 µl was added 1.5 µl of 6x loading dye (0.25% bromophenol blue; 0.25% xylene cyanol FF; 40% sucrose) were added. With the help of 10 µl pipette, the samples were loaded in individual wells along with the 50 bp size ladder (Fermentas, Lithunia, USA) as reference to determine the fragments size. The power pack was adjusted to 150 Volts/cm of run and the electrophoresis was run for 2-3 hours, after which the gel slabs were removed from the electrophoresis tank and visualized and documented under UV trans-illuminator in gel documentation system (Bio-Rad laboratories Inc., USA).

3.2.1.8 Linkage analysis

The genotypic and the phenotypic data of the F_2 population was analyzed for segregation distortion, if any, using chi-square (χ^2) test for goodness of fit as described earlier under the sub-heading statistical analysis under section 3.1.2.1. Linkage between the putatively linked marker and the target gene was estimated using genotypic data and the phenotypic data with the help of MAPMAKER ver. 3b software (Lander et al. 1987). Linkage was considered significant if the Logarithm of Odds (LOD) score was ≥ 3.0. The Kosambi mapping function (Kosambi, 1944) was used to convert recombination frequency into map distance between the marker and the target gene.

4 RESULTS

Mutation is an important factor in evolution for creating stable and heritable variations in living organisms. For more than 50 years, mutation breeding involving different mutagenic agents have been utilized to develop a large number of induced mutants which have been released as commercial cultivars in crops. These mutants have also been used as valuable genetic materials for functional genomics, breeding and for understanding the molecular basis of different traits including yield and superior grain quality in crop breeding. More than 1,00,000 mutants of rice have been produced by the rice researchers across the globe involving different mutagens and several mutant genes involved in plant architecture, grain quality, and disease resistance have been isolated, characterized and mapped in rice which induced by both types of mutagens, physical (irradiation, and fast neutron) and chemical (ethyl methane sulphonate, EMS). Development of various leaf colour mutants such as stripe, virescent, albino, chlorina, zebra and yellow variegated, in rice have been used as an interesting of research area which has helped in creating better understanding about the structure of chloroplast and chlorophyll biosynthesis in plants.

The present investigation entitled **"Genetic analysis and molecular mapping of *stripe* mutant in rice"** was conducted to understand nature and inheritance pattern of stripe mutant trait in the rice mutant, M507-1 and map the gene(s) governing this trait. To accomplish these objectives, the research involved the development of F_1 and F_2 populations derived from two different crosses namely, M507-1/Pusa 44 and M507-1/Nagina 22, using the stripe leaf mutant M507-1, developed by EMS induced mutation of an upland rice cultivar, Nagina 22. The results of the research study have been explained herewith in the ensuing sections to evidently understand characteristics and stripe leaf colour mutant of rice.

4.1 Genetics of stripe leaf trait in rice

The parental lines were thoroughly characterized for the leaf colour variations including the photosynthetic pigments as well as the leaf anatomy to make observations differentiating them from the mutant leaf trait. The research study for

understanding the inheritance of EMS-induced stripe leaf mutant trait in the mutant, M507-1, was conducted in the F_1 and F_2 population of two crosses, M507-1/Pusa 44 and M507-1/Nagina 22. The results from the observations documented on leaf colour with precise scoring and following presented segregation pattern with the probability limits for both the crosses.

4.1.1 Pigment contents among between wild type and mutant type rice lines

During the present investigation, differences in the leaf-color phenotype observed between wild (complete green) and mutant plants (Green with white stripes) were consistently exhibited in all leaves. The mutant, M507-1, exhibited white stripe in the leaves from seedling to maturity stage, whereas Pusa 44 and Nagina 22, showed complete green leaves till the maturity stage (Figure 1). To further characterize the stripe mutant, the photosynthetic pigments were measured using DMSO based method and SPAD chlorophyll meter at the different plant growth stages namely seedling, maximum tillering, booting and maturity stages, respectively (Table 4.1). The stripe leaf mutant, M507-1 had lower levels of total chlorophyll, chlorophyll a, chlorophyll b and carotenoids as compared to the wild type (Nagina 22) and Pusa 44 except at maturity stage, mutant type showed higher content of total chlorophyll, chlorophyll a, chlorophyll b and carotenoids than Pusa 44 (Figure 2). Overall, the white stripe in the stripe mutant leaf was found to have a direct impact on the chlorophyll content. These observations indicated that the stripe mutant exhibited obvious defective mechanism of chlorophyll biosynthesis throughout its whole life cycle, from seedling to maturity.

4.1.2 Variations in transverse leaf sections of stripe mutant and wild plant leaves

Defective mechanism in the synthesis of chlorophyll is usually related with abnormal leaf colour development as discovered in the stripe leaf mutant. To further study the effect of white stripe mutation on the development of leaf and internal anatomy, transverse sections of leaves in wild type (Nagina 22 and Pusa 44) and mutant, M507-1 were observed through microtome sectioning. Number of bundle sheath, minor veins between two major veins clearly differentiated mutant (M507-1) from the wild type (Nagina 22 and Pusa 44) leaf structures under light microscope (Figure 3).

Pusa 44 **M507-1** **Nagina 22**

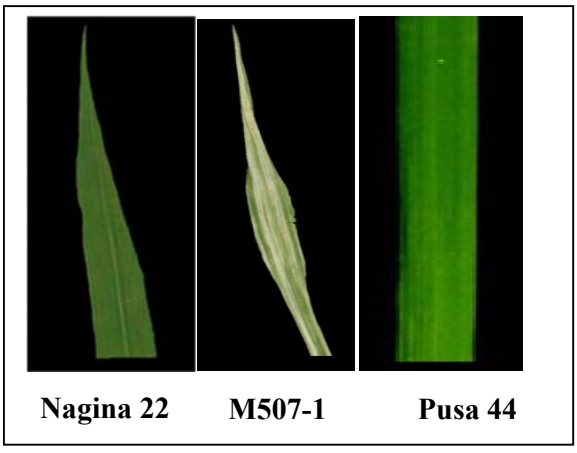


Figure 1. Phenotypic variation in the leaf colour of the normal green coloured leaves of 'Nagina 22' (wild type complete green), 'M507-1' (stripe leaf mutant showing white stripe) and 'Pusa 44' (complete green).

In the stripe leaf mutant, M507-1, the number of bundle sheath was comparatively lower than wild type plant leaves (Nagina 22 and Pusa 44). Additional, M507-1 also showed lower number of minor veins between two major veins in the leaves as compared to wild type leaves (Nagina 22). The starch grains in mesophyll tissues were also lower in the mutant as compared to wild type leaves. Variations in leaf structures between mutant (M507-1) and wild (Nagina 22 and Pusa 44) plants exhibited the formation of lower content of photosynthetic pigments in mutant plants (M507-1).

4.2 Segregation for leaf colour trait in the F_2 population

4.2.1 Inheritance of stripe leaf trait in the F_2 population, M507-1/Pusa 44

A total of 333 individual plants of the F_2 population from the cross between stripe leaf mutant, M507-1 and complete green leaved, Pusa 44 were phenotyped for leaf colour. Based on the leaf colour, the F_2 population was classified into two categories namely plants with normal green coloured leaves and stripe leaf. There were no intermediate types showing differential expression of the stripe leaf phenotype. It was observed on basis of leaf colour, that out of 333 plants, 281 plants possessed completely green (CG) like Pusa 44, while 52 plants were striped leaves similar to the mutant, M507-1 (Table 4.2). The frequency distribution of the F_2 population with respect to leaf colour namely complete green and mutant white stripe plants showed that the stripe leaf colour is governed by two genes acting in inhibitory epistatic manner. It showed a good fit to segregation ratio of 13:3 (p= 0.1406) (Table 4.2), indicating that the leaf colour trait in the cross M507-1/Pusa 44 was controlled by two genes exhibiting inhibitory epitasis (Figure 4).

Table 4.1. Variation in photosynthetic pigment contents in the stripe leaf mutant (M507-1) and the normal green leaved wild type, Nagina 22 and Pusa 44

Genotype	Total Chl	Chl a	Chl b	Carotenoids
A. Seedling stage				
MG507-1	3.47	2.92	0.56	0.76
Nagina 22	5.34	4.51	0.84	1.11
Pusa 44	6.69	5.60	1.11	1.26
B. Maximum Tillering stage				
M507-1	2.53	2.22	0.31	0.63
Nagina 22	6.33	5.53	0.8	1.30
Pusa 44	6.93	5.94	1	1.27
C. Booting stage				
M507-1	1.57	1.30	0.27	0.37
Nagina 22	3.75	3.15	0.60	0.72
Pusa 44	3.51	5.94	0.56	0.70
D. Maturity stage				
M507-1	1.31	1.06	0.25	0.36
Nagina 22	1.85	1.49	0.37	0.46
Pusa 44	1.22	1.01	0.21	0.32

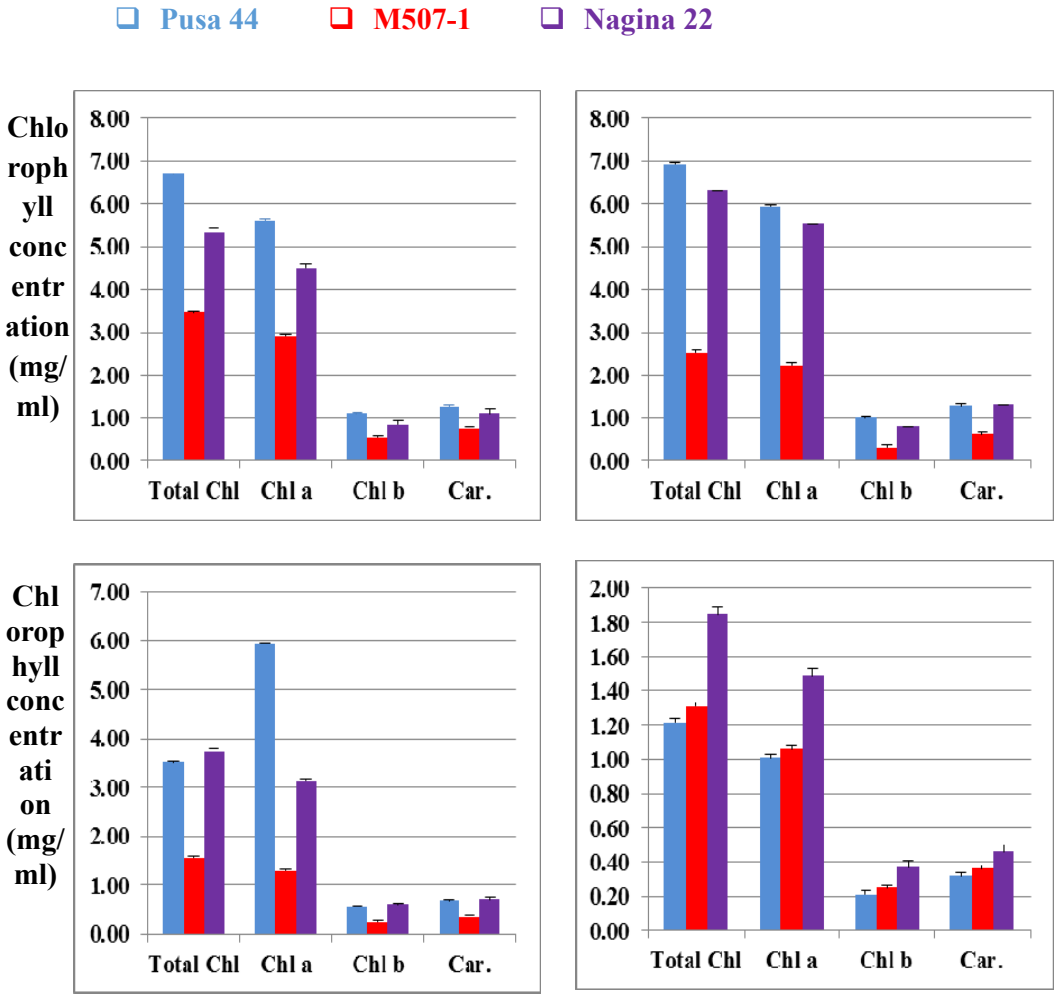


Figure 2. Variation in the photosynthetic pigments of the complete green leaves of wild-type, Nagina 22 and Pusa 44 and the white stripe leaves of mutant, M507-1 at different plant growth stages namely (a) seedling, (b) maximum tillering, (c) booting and (d) maturity, respectively.

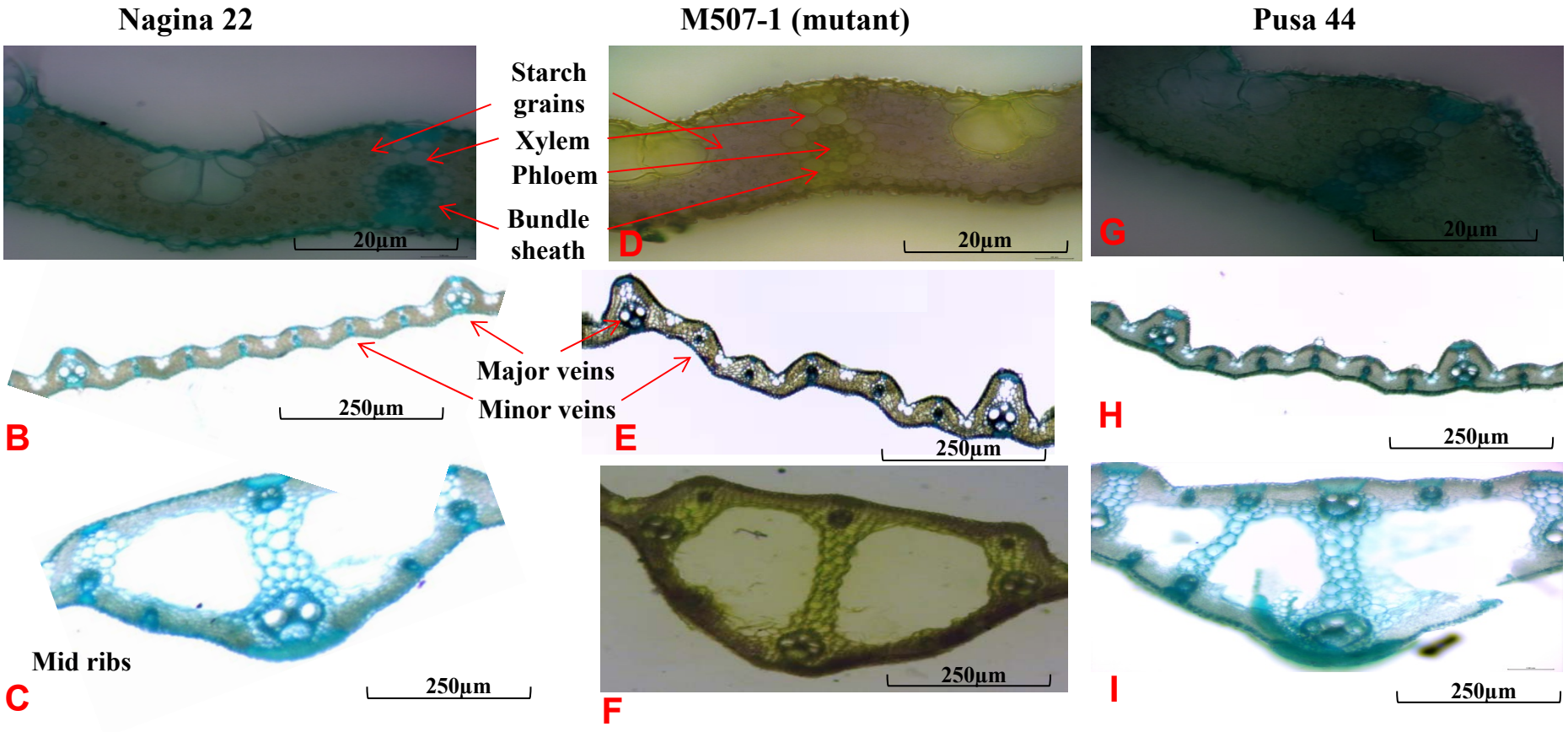


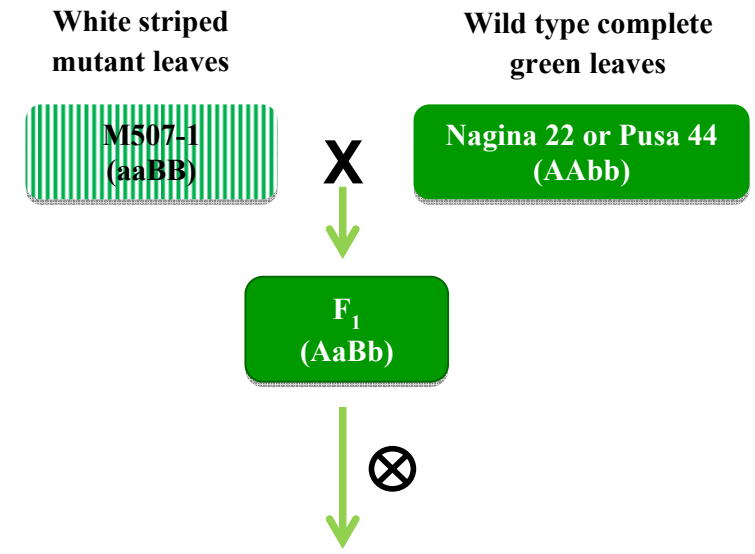
Figure 3. Transverse section of the leaves of the wild type complete green leaved, Nagina 22 (A, B, C), white striped leaved mutant M507-1 (D, E, F) and the green leaved Pusa 44 (G, H, I) as observed under light microscope.

4.2.2 Inheritance of stripe leaf trait in the F_2 population of M507-1/Nagina 22

A total 369 plants were evaluated for leaf colour from the cross, M507-1/Nagina 22 and grown along with the parental lines M507-1 and Nagina 22 during *Kharif* 2017 at ICAR-IARI, New Delhi. The leaf colour was phenotyped in 369 individual plants in the F_2 population, based on which two categories were observed namely, completely green and stripe leaves with white stripe. Out of 369 plants, a total of 309 and 60 plants were found to produce completely green and striped leaves, respectively (Table 4.2). The frequency distribution of F_2 population for leaf colour in this cross also suggested that the inheritance of stripe leaf colour was governed by a pair of genes acting in inhibitory epistatic manner. The frequency distribution for the leaf colour trait in this cross also showed a good fit of 13:3 ratio (p= 0.0017), indicating that the stripe leaf trait in this cross M507-1/Nagina 22 was controlled by two genes exhibiting inhibitory epitasis (Figure 4).

Table 4.2. Segregation analysis of stripe leaf mutant trait in F_2 population from the crosses M507-1/Pusa 44 and M507-1/Nagina 22

Crosses	Total no. of plants	No. of F_2 plant		Expected ratio	χ2-value	p-value
		Normal green leaves	Stripe leaves			
M507-1/ Pusa 44	333	281	52	13:3	2.172	0.1406
M507-1/ Nagina 22	369	309	60	13:3	9.899	0.0017



♀ / ♂	AB	Ab	aB	ab
AB	AABB	AABb	AaBB	AaBb
Ab	AABb	AAbb	AaBb	Aabb
aB	AaBB	AABb	aaBB	aaBb
ab	AaBb	Aabb	aaBb	aabb

Figure 4. A schematic figure showing the inheritance of the complete green and white stripe leaf colour trait governed by two genes exhibiting inhibitory epitasis in two different crosses namely, M507-1/Nagina 22 and M507-1/Pusa 44, respectively.

4.3 Molecular mapping of the mutant gene governing white stripe leaf colour trait in mutant line, M507-1

4.3.1 Polymorphism survey between parental lines M507-1/Pusa 44 and M507-1/ Nagina 22

A total of 1114 simple sequence repeat (SSR) markers across a set of 12 chromosomes were used for polymorphism survey between the parental lines, M507-1, Pusa 44 and Nagina 22 to detect polymorphic markers. Out of these markers, 216 markers including three markers series, RM, RGNMS and HvSSR, were found to be polymorphic between M507-1 and Pusa 44 (Figure 5) exhibiting 19.39% polymorphism, while no marker was found polymorphic between M507-1 and Nagina 22 (Figure 5). Therefore, molecular analysis for mapping of mutant genes governing stripe leaf colour trait in the mutant was conducted employing Bulked Segregant Analysis (BSA) strategy using the individuals of F_2 population obtained from the cross between M507-1 and Pusa 44. A diagrammatic graphical genotype of the 216 polymorphic SSR markers on the 12 rice linkage groups is represented in Figure 6.

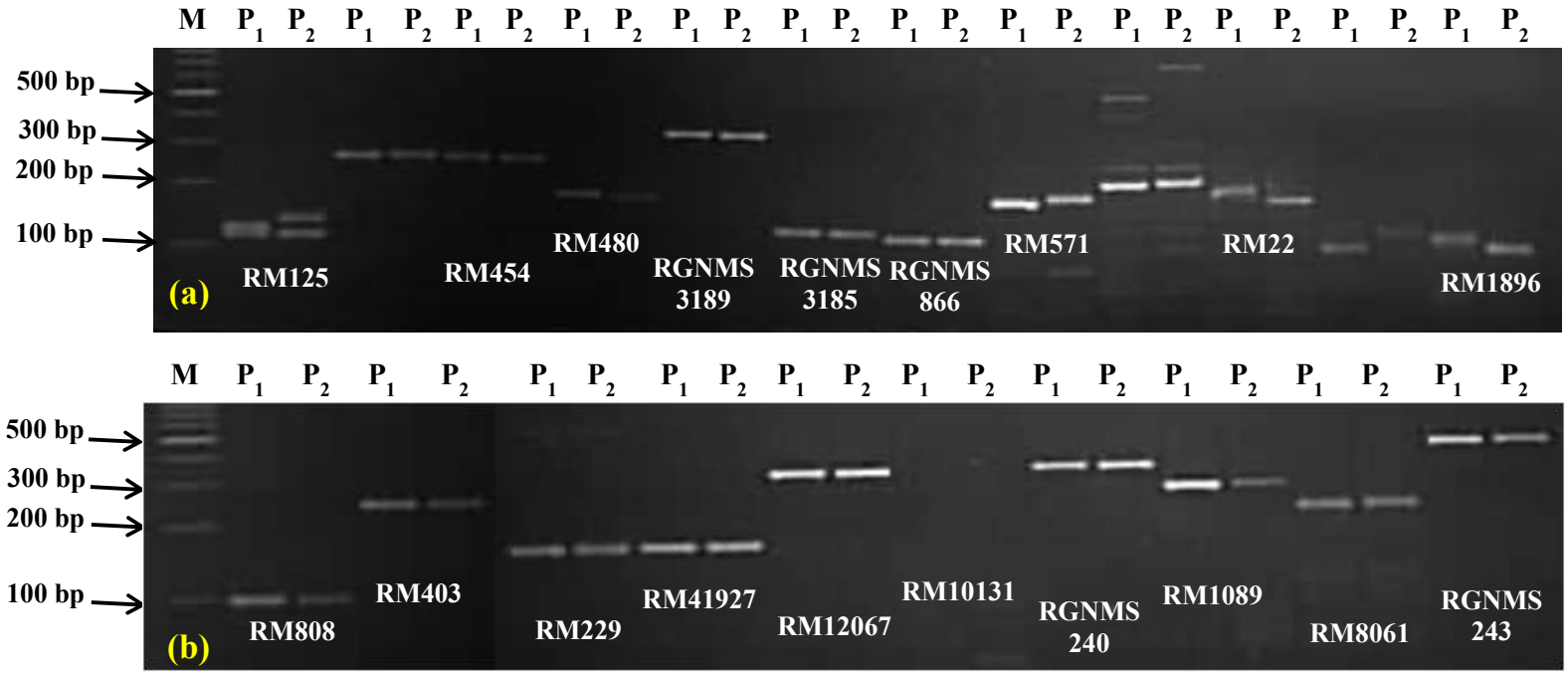


Figure 5. Representative gel picture of the parental polymorphism survey showing amplification profile between the parental lines (a) M507-1 (P_1) and Pusa 44 (P_2) and (b) M507-1 (P_1) and Nagina 22 (P_2) using SSR markers, M = 100 bp DNA ladder.

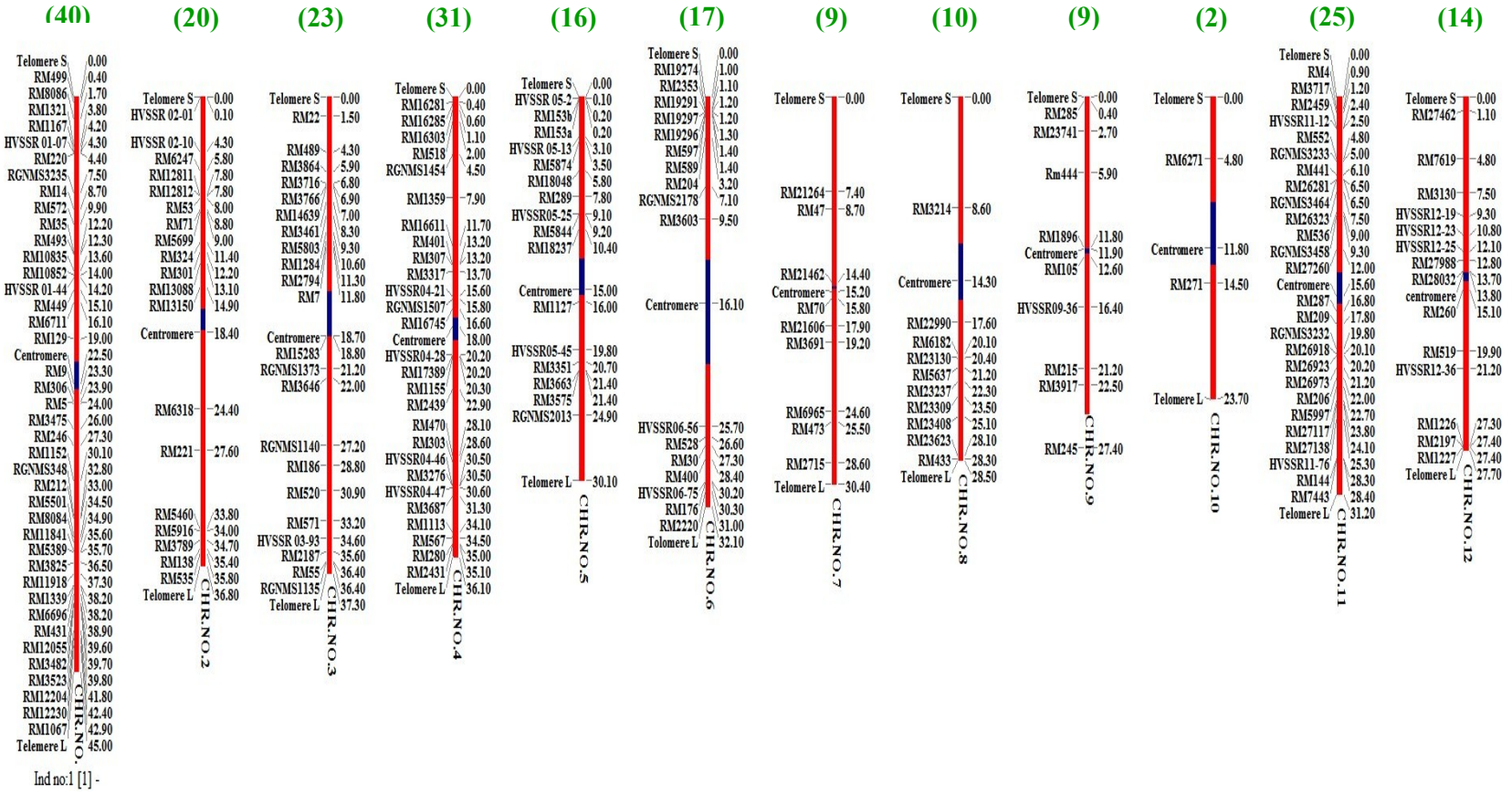


Figure 6. A graphical genotype showing the genomic distribution of the 216 polymorphic SSR markers between the parental lines, Pusa 44 and M507-1 in the 12 rice linkage groups.

4.3.2 Bulked Segregant Analysis

In the present study, it was observed in the F_2 population that mutant white striped leaves exhibited segregation in 13:3 ratio showing that the stripe leaf colour is digenic with inhibitory epistasis in both F_2 populations (M507-1/Pusa 44 and M507-1/Nagina 22). Therefore, a modified two step Bulked Segregant Analysis (BSA) strategy was adopted to identify markers putatively linked with the genes governing stripe leaf colour trait in rice (Figure 7). Two different DNA bulks, one of wild type bulk from 10 plants showing completely green leaf colour and the other mutant bulk from 10 plants showing white stripe leaves were constituted for the first BSA (BSA-I) from F_2 population based on their leaf colour behavior. Further, a total of 216 polymorphic markers were screened in both wild and mutant bulks with parental DNAs (Pusa 44 and M507-1) to detect putatively linked markers to trait of interest (Figure 9). In first BSA, one marker on chromosome 5 namely, 'HvSSR05-02' showed heterozygosity in the wild type bulk (completely green leaved plants), while homozygosity was observed in mutant white striped leaf bulk (Figure 9) which revealed that the marker HvSSR05-02 may be putatively linked to gene which controlled complete green leaf colour trait.

Based on the genotyping of F_2 individuals with putatively linked marker, HvSSR05-02, the plants homozygous for the recessive allele of the HvSSR05-02 were selected for the BSA-II and another two DNA bulks were constituted in which, one comprised of DNA of 10 individuals showing wild type normal green leaves while the other comprised of DNA of 10 individuals showing mutant white striped leaf colour, to identify markers putatively linked to the second gene controlling mutant white stripe leaf colour in M507-1. As in earlier BSA, all 216 polymorphic markers were again screened in the BSA-II with these two bulks (Figure 10). In the second BSA, three markers namely, 'RM153a, RM153b, RGNMS2013' on chromosome 5, showed the expected amplification pattern between both bulks along with parents. As per banding pattern, wild type completely normal green bulk showed homozygosity, while heterozygosity was observed in the white striped mutant leaf bulk (Figure 11 and 12) which indicated that the all these markers 'RM153a, RM153b, RGNMS2013' may be putatively linked to second gene which expressed mutant white stripe leaf colour trait.

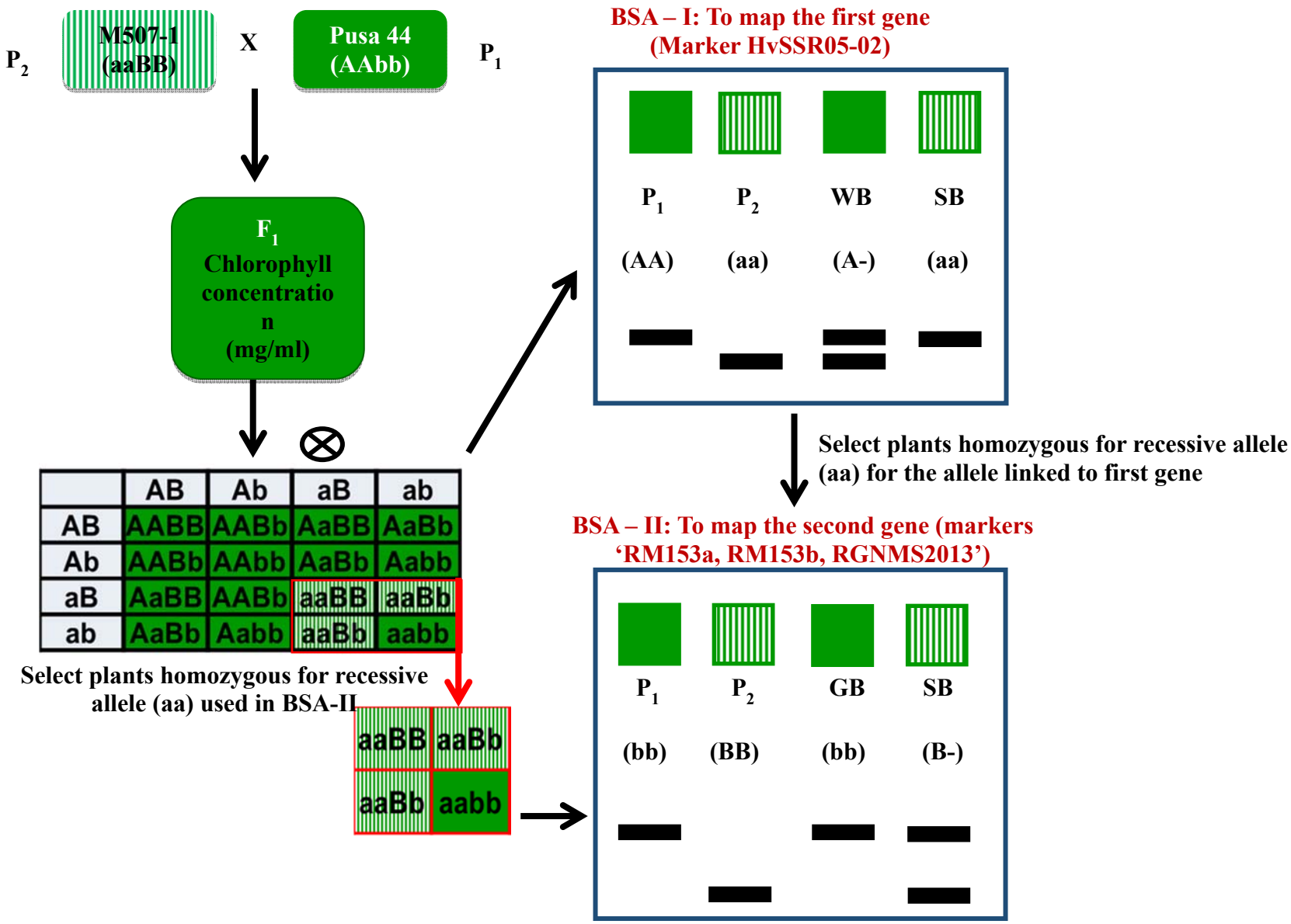


Figure 7. A modified two step Bulked Segregant Analysis (BSA) strategy for mapping genes governing stripe leaf colour trait in rice.

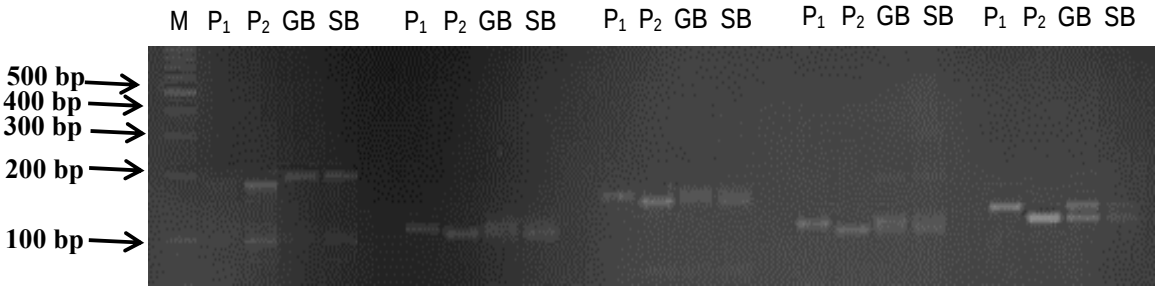


Figure 8. Bulked Segregant Analysis I with the polymorphic markers between the parents, Pusa 44 (P_1) and M507-1 (P_2) showing monomorphism between both the green leaved bulk (GB) and white stripe leaved bulk (SB), M=100 bp DNA ladder.

Code	Parent/ Bulk	Phenotype	Genotype
P_1	Pusa 44		AA_{bb}
P_2	M507-1		aa_{BB}
GB	Green bulk		$A\text{-}_{B\text{-}}$
SB	Stripe bulk		$aa_{B\text{-}}$

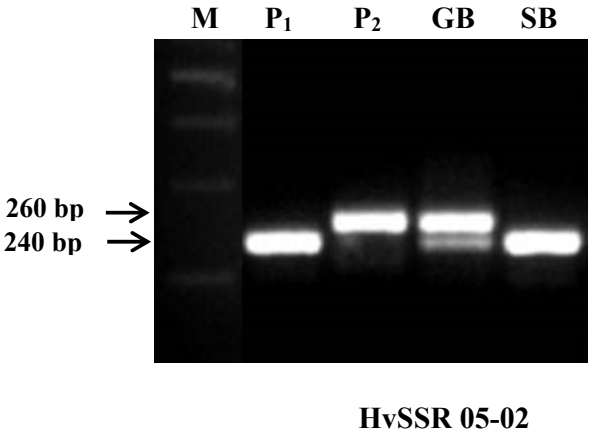


Figure 9. Bulked Segregant Analysis I: The hyper variable SSR marker, HvSSR05-02 showing polymorphism between the parents, Pusa 44 (P_1) and M507-1 (P_2) as well as contrast between the green leaved bulk (GB) and white stripe leaved bulk (SB), identified to be putatively linked with one of the genes controlling stripe leaf colour, M=100 bp DNA ladder.

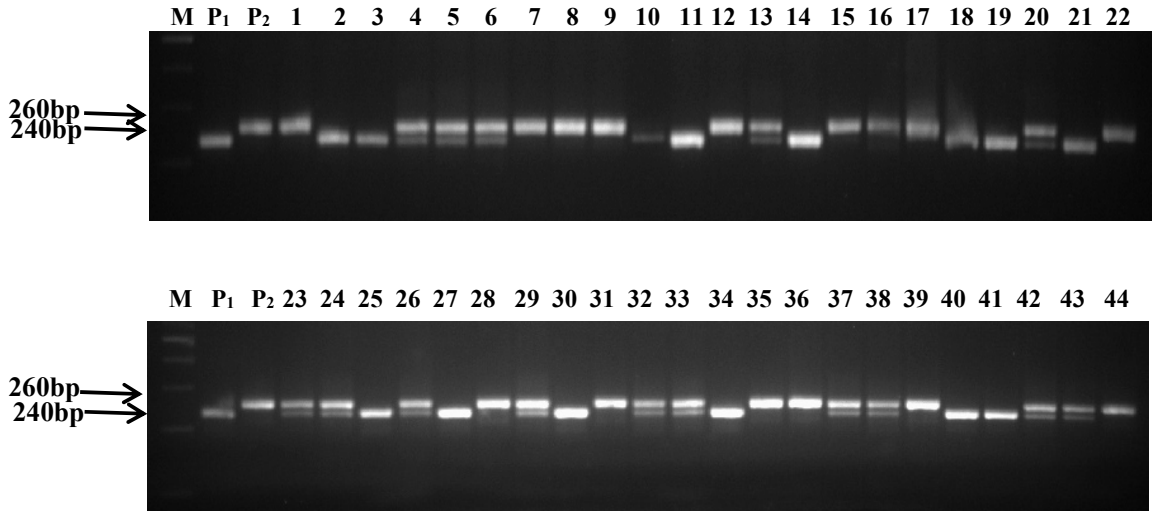


Figure 10. A representative gel picture showing the genotyping of the F_2 individuals using HvSSR05-02 identified to be putatively linked with one of the genes controlling stripe leaf colour in Bulked Segregant Analysis I, M=100 bp DNA ladder.

Code	Parent/ Bulk	Phenotype	Genotype
P_1	Pusa 44		AAbb
P_2	M507-1		aaBB
GB	Green bulk		aabb
SB	Stripe bulk		aaB-

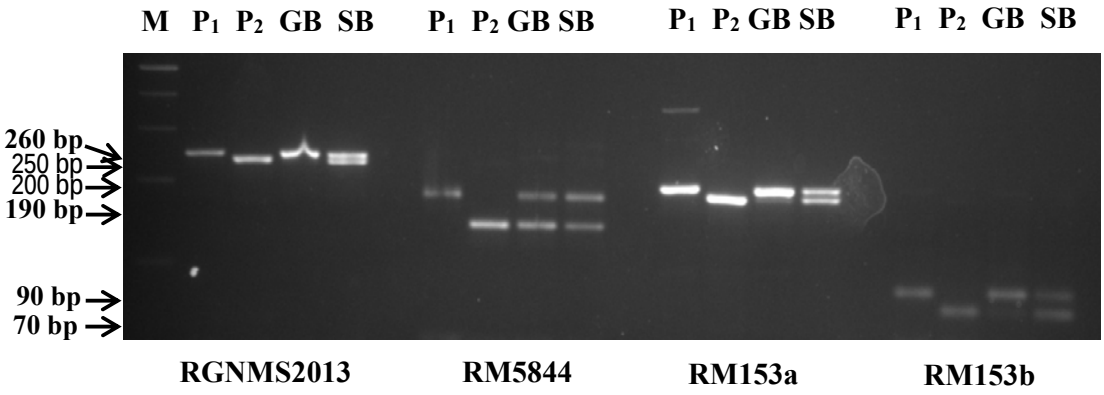


Figure 11. Bulked Segregant Analysis II: The SSR marker, RM153a, RM153b, RGNMS2013 showing polymorphism between the parents, Pusa 44 (P_1) and M507-1 (P_2) as well as contrast between the green leaved bulk (GB) and white stripe leaved bulk (SB), ladder identified to be putatively linked with the second gene controlling stripe leaf colour, M=100 bp DNA ladder.

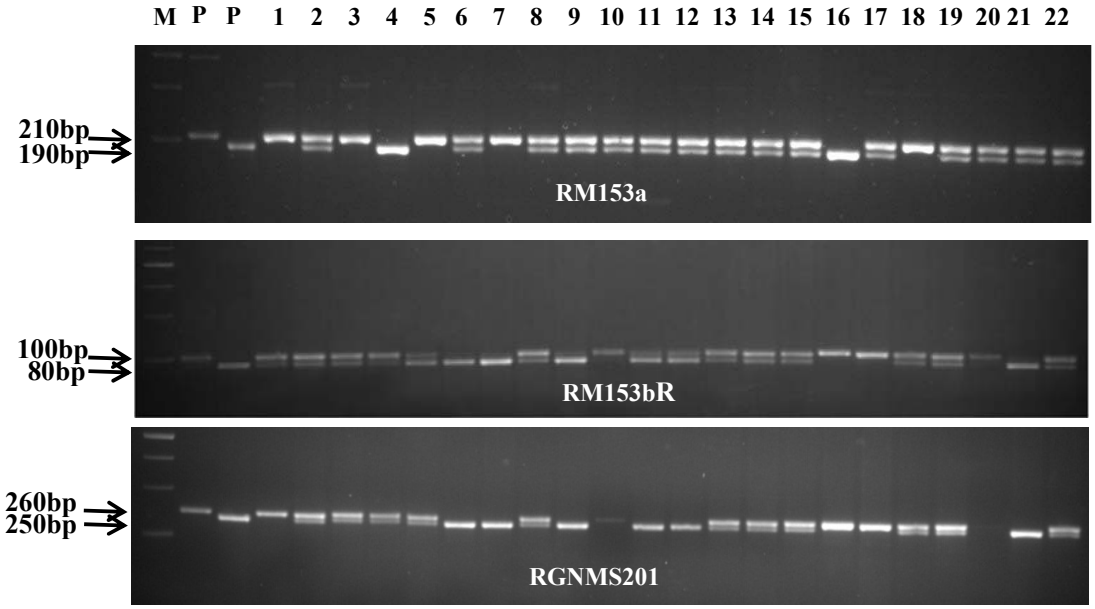


Figure 12. A representative gel picture showing genotyping of the F_2 individuals using RM153a, RM153b, RGNMS2013 identified to be putatively linked to the second gene governing stripe leaf trait in Bulked Segregant Analysis II, M=100 bp DNA ladder.

4.3.3 Genotyping of the F_2 individuals with putatively lined markers

The genotyping of a subset of 157 F_2 individuals from the cross M507-1/Pusa 44 was undertaken with the putatively linked markers, HvSSR05-02 (Figure 10) identified from BSA-I and RM153a, RM153b, RGNMS2013 (Figure 12) detected from BSA-II used for linkage analysis to identify the linkage between the putatively linked markers and the genes governing the stripe leaf trait in rice mutant, M507-1.

4.3.3.1 Segregation analysis of the phenotypic and genotypic data

The phenotypic data of F_2 plants from the cross M507-1/ Pusa 44 was tested for goodness of fit using χ^2 test against the expected ratio of 13:3 for wild complete green type and stripe mutant plants. The observed frequencies fitted well with the expected ratio 13:3 with a p-value of 0.1406 (Table 4.2). The goodness of fit the genotypic segregation of 157 plants in the F_2 population for the marker loci HvSSR05-02 and RM153a, RM153b, RGNMS2013 were tested using χ^2 test against the expected ratio 1:2:1. The observed number of plants, expected genetic segregation ratio and probability of fit for each marker was presented in the Table 4.5. It is evident from the results obtained ratio fitted well with the expected ratio with respect to HvSSR05-02, RM153a, RM153b, RGNMS2013 markers with a p-value of 0.750, 0.750, 0.808 and 0.168, respectively.

Table 4.3. Segregation analysis of the genotypic data of the markers, HvSSR05-02 and RM153a, RM153b, RGNMS2013 in the F_2 population from the cross M507-1/ Pusa 44

M507-1/ Pusa 44	Total no. of plants	No. of F_2 plants			Expected segregation ratio	χ^2 - value	p-value
		A (AA)	H (Aa)	B (aa)			
HvSSR05-02	157	38	83	36	1:2:1	0.567	0.750
RM153a	157	38	83	36	1:2:1	0.567	0.750
RM153b	157	39	82	36	1:2:1	0.427	0.808
RGNMS2013	157	43	85	29	1:2:1	3.573	0.168

4.3.4 Linkage analysis

To map one of the gene governing stripe leaf colour trait in the mutant, M507-1, linkage analysis was carried out using phenotypic data on mutant and genotypic data from SSR marker, HvSSR05-02 identified to be putatively linked to one of the genes (*Stp1*) governing stripe leaf trait through BSA-I, The genotypic data on a subset of 157 individuals of F_2 population from the cross, M507-1/Pusa 44 was used for linkage analysis using MAPMAKER ver.3b. HvSSR05-02 was mapped at a distance of 21.7 cM using linkage analysis conducted using Kosambi function (Figure 13 and 14).

To map the second gene governing stripe leaf colour trait in the mutant, M507-1, linkage analysis was carried out using phenotypic data on mutant and genotypic data from SSR markers, RM153a, RM153b, RGNMS2013 generated on a subset of 157 individuals of F_2 population from the cross, M507-1/Pusa 44 using MAPMAKER ver.3b. The SSR markers, RM153a, RM153b, RGNMS2013 were mapped at a distance of 8.6 (LOD 10.03), 11.9 (LOD 7.24) and 43.8 cM (LOD 2.80) using linkage analysis conducted using Kosambi function (Figure 13 and 14).

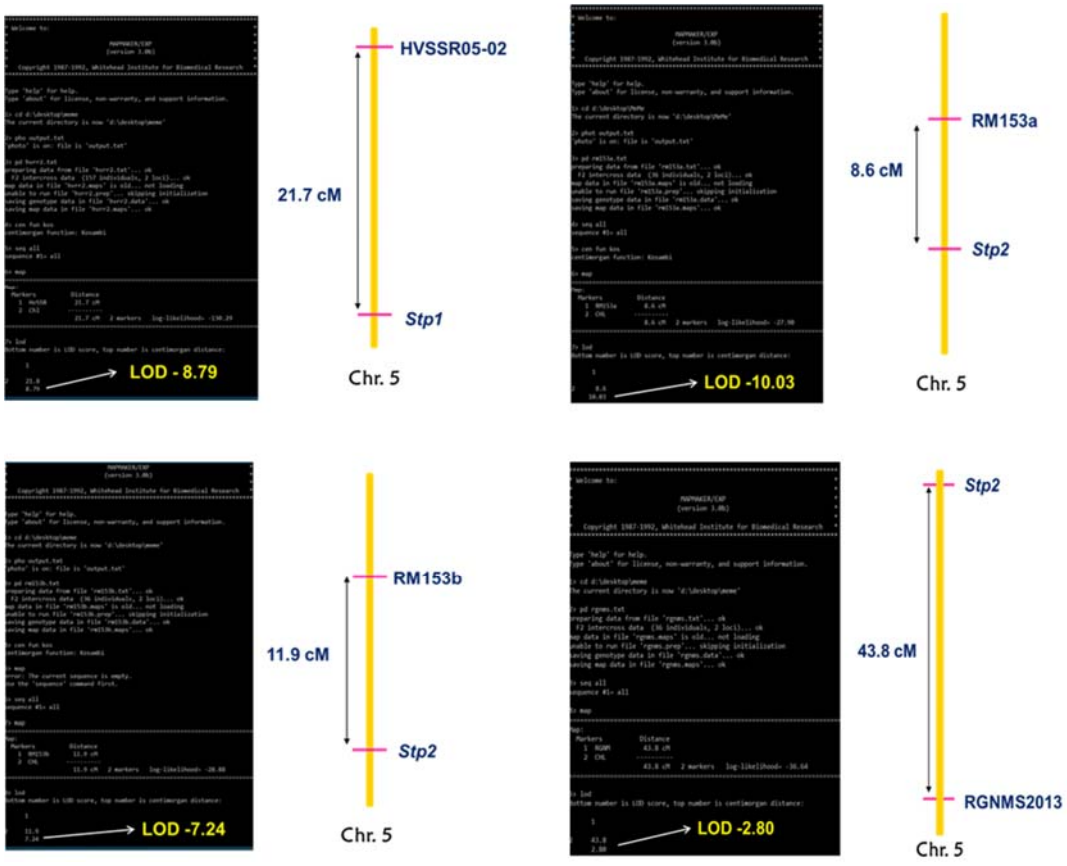


Figure 13. Linkage maps showing the linkage of SSR markers linked to the two genes, *Stp1* and *Stp2* genes governing leaf colour in white stripe leaf mutant, M507-1.

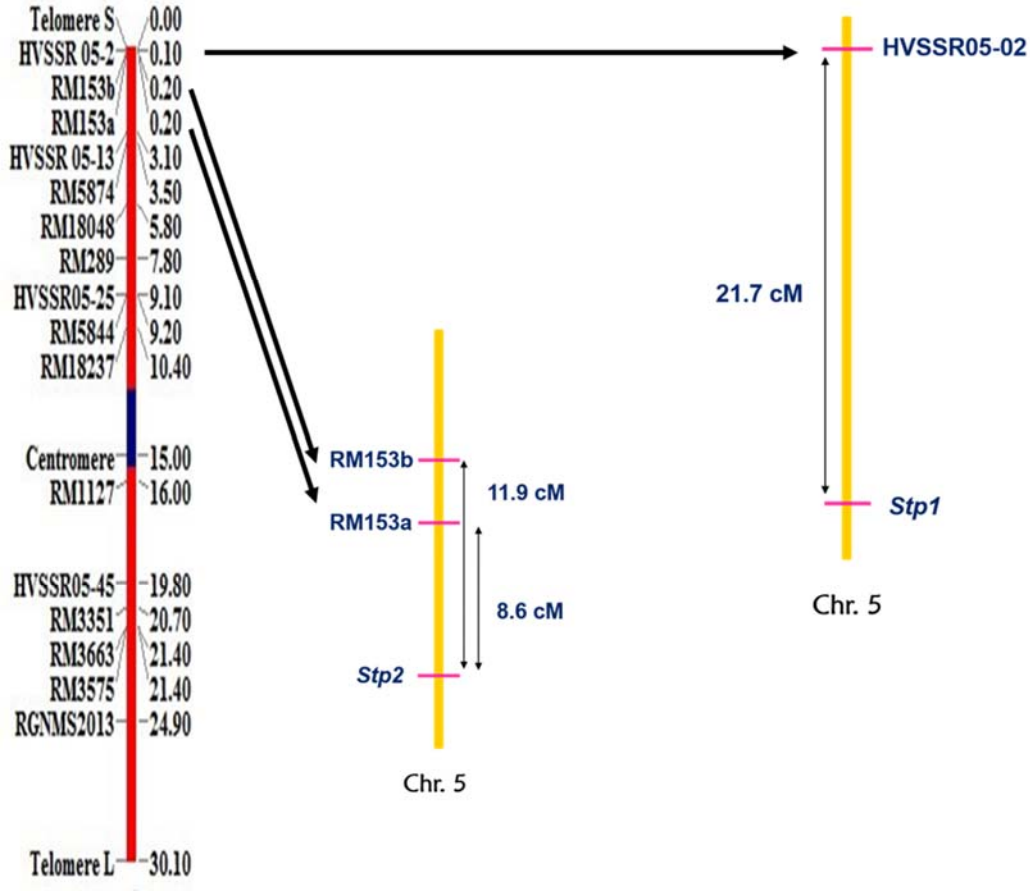


Figure 14. Linkage map showing the physical position and linkage of SSR markers linked to the two genes, *Stp1* and *Stp2* genes governing leaf colour in white stripe leaf mutant, M507-1.

5 DISCUSSION

Rice (*Oryza sativa* L.) is an important food crop consumed by more than 50% of the world's population. There is an increasing demand for rice to feed the burgeoning population despite the challenges of climate changes, biotic and abiotic stresses (Xuan and Khang, 2018). Therefore, rice researchers are focused on improving its productivity to ensure food security and sustainability through the development of rice cultivars with higher yield, durable resistance, desirable grain quality, and good adaptation (Xing and Zhang, 2010). However, the development of new rice cultivars with new desired traits generally takes 8–10 years or more, which involves laborious work and need expansive expenditure (Xuan et al., 2019).

Rice has been the model crop for plant researchers for the past decade due to the availability of vast array of genomic and genetic resources in the form of mutants, which can be utilized for its improvement. Mutation breeding has been adopted for development of improved cultivars/varieties without compromising the agronomic and quality characteristics through creation of useful variations in the form of mutations through induced mutagenesis. Through classical mutagenesis, more than 3000 mutant varieties have been developed in more than 200 plant species over the past six decades (Kurowska et al., 2012). Out of this, around 400 mutant varieties have been developed in rice alone through mutation breeding. Among the rice mutants, the abnormal leaf colour mutants are considered novel genetic resource for understanding the basic mechanisms involved in chloroplast development as well as chlorophyll biosynthesis. A large number of leaf color mutants have been created in several crops including rice (Hu et al., 1981), maize (Lin and Yu, 1995), barley (Rudoi and Shcherbakov, 1998), and wheat (Marco et al., 1989) through use of physical and chemical mutagens. Among the chemical mutagens, EMS has been sued in developing leaf color mutants (Wang et al., 2014) as well as other useful mutants for rice improvement (Lee et al., 2003). In rice, an array of more than 180 leaf color mutants have been characterized and as many as 80 genes controlling leaf colour variations disperse in all the 12 chromosomes have been isolated (Deng et al., 2014; Sun et al., 2017). Most of leaf colour variations have been reported to be governed by a pair of recessive nuclear genes (Qian et al., 1996; Wang et al., 2008; Xie et al., 1995).

A leaf colour mutant, M507-1, was developed through EMS induced mutagenesis through which as many as 370 EMS mutants were generated and characterized in the background of Nagina 22, a popular drought- and heat-tolerant upland cultivar (Mohapatra et al., 2014; Amitha et al., 2016). With the above background, the present research entitled **"Genetic analysis and molecular mapping of s*tripe* mutant in rice"** was carried out at Division of Genetics, ICAR-IARI, New Delhi. In this study, the white stripe leaf mutant, M507-1, displaying characteristic white stripes along the entire leaf, stably inherited across the generations was characterized through quantification of photosynthetic pigments and the leaf anatomy while molecular mapping was attempted to map the gene(s) governing the stripe leaf mutation in this mutant genotype. The salient findings of the research study are discussed in the following section in light of the literature on leaf colour variation in rice.

5.1 Leaf colour variation in stripe leaf mutant, M507-1

In the present study, the mutant, M507-1, developed from Nagina 22 through EMS induced mutagenesis exhibited 'white stripe' leaf colour from seedling to maturity stage. In rice, different leaf color mutants, including light green, yellow, albino, purple, spotted, zebra, shaded green and reversible whitening at different growth stages, have been reported to be generated by EMS and irradiations (Li et al., 2018). The stripe leaf phenotype is characterized by a white sectored leaf in the longitudinal direction, which vary in size as well as numbers based on the loci governing them, whereas, zebra mutant shows a white or yellow transverse band varying in its size, frequency, colour as well as the developmental stage of plant growth across the green leaves (Kurata et al., 2005). Among all of them the most commonly identified leaf colour is yellowish-green mutant phenotype, that is generally observed at the seedling stage (Goyal et al., 2019), which then turns back to a normal green colour at maturity (Thang et al., 2010; Deng et al., 2014), while in some cases it is observed all along right from seedling till maturity stage (He et al., 2018). Chemical mutagens like EMS creates point mutations, which alters the leaf colour at seedling stage to "zebra-type stripes" with permutation of yellow and white in whole plant (Wang et al., 2009). The occurrence of zebra-type stripe mutant has been reported to be temperature dependent (Xie et al., 1995), while that of white-striped leaf colour appearing after the second leaf growth and its sensitivity to

temperature is also reported in rice (Xu et al., 2010). Leaf colour variations in different mutants can serve as effective phenotypic markers to differentiate the hybrids and its genetic purity in hybrid breeding (Du et al., 2009). Alternately, these mutants also serve as ideal genetic material for unraveling the physiological and metabolic processes including chloroplast structure, function and development, the chlorophyll metabolism, leaf color manipulation and, resistance to diseases.

5.2 Characterization of photosynthetic pigments and leaf anatomy in the stripe leaf mutant

The total chlorophyll, chlorophyll a, chlorophyll b and carotenoids in the stripe leaf mutant, M507-1, was observed to be low as compared to the wild type plants with normal green leaves at all the growth stages except maturity stage, where the mutant exhibited higher content of total chlorophyll, chlorophyll a, chlorophyll b and carotenoids compared to the wild type. Lower content of pigments observed in the mutant leaf-colour could be attributed to the degradation of chlorophyll or the breakdown of chlorophyll biosynthetic pathway, which has been reported in rice by Masuda et al., 2003. The chlorophyll content of white striped leaf mutant (*wsl1*) developed through EMS induced mutagenesis of the *japonica* cultivar, Nipponbare showed significantly lower chlorophyll content than that of wild-type during the fourth leaf stage, tillering stage and booting stage (Sheng et al., 2013), which has proved that the reduced chlorophyll content in the EMS-induced mutant rice could be due deactivation of the chlorophyll synthesis genes. Such decrease in the pigment contents in the EMS-induced mutants have been reported in various other crops exhibiting variation in leaf colour, such as soybean (Walker et al., 2018) and wheat as well (Hamblin et al., 2014).

Variations in leaf structures between mutant and wild type plants as well as the formation of photosynthetic pigments in mutants due to defective chlorophyll synthesis mechanism could lead to abnormal leaf development as pronounced as leaf-color variations in plants. Changes in leaf colour have been attributed to the mutations which affects chloroplast development and chlorophyll biosynthesis as well as biodegradation pathways (Wu et al. 2007; Yoo et al. 2009; Sato et al., 2009). In present study, the white stripe leaf mutant was observed for any variation in the leaf structure development through microtome sections of the transverse sections of leaves in wild and mutant plants. Transverse sections of the leaves of the stripe leaf mutant

and normal green leaved wild type plants were observed under light microscope, which showed that the number of bundle sheath were less in the mutant, M507-1 as compared to the wild type plant leaves (Nagina 22 and Pusa 44). Additionally, the number of minor veins between two major veins was also observed to be low in the mutant leaves (M507-1) as compared to wilt type leaves (Nagina 22). More number of starch grains in the mesophyll tissues was also detected in the wild type leaves as compared to the mutant leaves. EMS–induced white stripe mutant rice '*wsl*1' was observed to show degenerated thylakoids and reduced numbers of chloroplast, grana lamella and reduced starch granules compared to the leaf structure of with wild type (Sheng et al., 2013). Tan et al., (2019) also observed significant reduction in the number of chloroplasts in the mesophyll cells as well as the overall volume of the chloroplasts in transverse sections of yellow-green leaf of an *indica* restorer line of rice, Xinong 1B, due to EMS mutagenesis. Additionally, some of the mutants showed chloroplasts with disrupted structural integrity and substantially larger starch granules, whereas some of them showed normal lamella structure and typical starch granules. The presence of excessive starch grains in the chloroplast not only damages the thylakoid, but also blocks light penetration and directly obstructs light absorption by the photosynthetic membrane, leading to obstruction of the photosynthetic system (Woo et al., 2001).

5.3 Inheritance of white stripe leaf in M507-1

In the present study, the segregation the stripe leaf mutant and the normal green leaf wild type showed a good fit to non-Mendelian segregation ratio of 13:3. It indicated that the leaf colour trait in the cross M507-1/Pusa 44 was controlled by two genes exhibiting inhibitory epitasis (9:3:3:1). Chemical mutagens are ideal for inducing dominant mutant alleles, while physical mutagens have been found to create recessive mutations (Wang et al., 2014). White stripe leaf phenotype in the *wsl3* mutant was controlled by a single recessive nuclear gene and segregated with 3 green to 1 white stripe ratio in the F_2 populations from the reciprocal crosses, *wsl*3/93-11 and 93-11/*wsl3* (Zhou et al., 2016). Reversible green albino as well as yellow leaf mutations controlled by a single recessive gene has been reported in rice (Zhao et al., 2006). A white lesion mimic leaves1 (*wlml1*) mutant derived through EMS induced mutation was characterized using an F_2 population of the *indica* rice cultivar Taichung Native1 (TN1), which showed Mendelian segregation pattern of 3:1 suggesting its

inheritance to be controlled by a recessive nuclear gene (Chen et al., 2018). Very recently, another recessive gene, *ygl14* has been identified, which produce a typical yellow-green striped leaf, which is governed by a single recessive gene (Tan et al., 2019). All the leaf colour mutations reported till date have been found to be monogenic recessive in nature, while the leaf striped colour mutation in M507-1 have been observed to be governed by two genes acting in inhibitory epistatic manner. This is the first of its kind report for leaf colour mutation in rice.

5.4 Molecular mapping of striped leaf mutation in M507-1

A large number of genes governing leaf-color mutation in rice have been mapped onto different chromosomes and most of them have been cloned. Genes such as stripe (*st2*) (Xu et al., 2014), white-stripe leaf (*wsl3*) (Zhao et al., 2016) and yellow-green leaf (*YGL8*) (Zhu et al., 2016) on chromosome 1; white stripe leaf (*WSL4*) on chromosome 2 (Wang et al., 2017); white stripe leaf (*st10*) on chromosome 3 (He et al., 2011); yellow-green leaf (*ygl1*) on chromosome 5 (Wu et al., 2007); white stripe (*st1-2*) (Chen et al., 2015) and white stripe (*st1*) (Yoo et al., 2009) on chromosome 6; white striped leaf (*wsl1*) on chromosome 9 (He et al., 2011); green-white-green leaf (*GWGL*) (Li et al., 2012) and white stripe leaf (*WSL12*) (Ye et al., 2016) on chromosome 12 have been reported in rice. In the present study, epistatic inheritance was observed in segregation pattern with the ratio of 13:3 in F_2 population, which indicated that the stripe leaf colour trait in the EMS induced mutant, M507-1 is governed by two different genes. Among the two populations developed from the crosses, M507-1/Nagina 22 and M507-1/Pusa 44, the cross with Nagina 22 could not be used for mapping the genes using SSR markers, as the mutant, M507-1 is an EMS induced mutation from Nagina 22. As EMS mainly induces point mutations, the possibility of obtaining polymorphisms using SSR markers, whose polymorphism is based on the number of tandem repeats, it is not expected to show any polymorphism with its own wild type from which the mutant has been generated. The analysis of polymorphism between Nagina 22 and M507-1 showed that all the SSR markers from chromosome 1 were monomorphic in nature.

Therefore, the mapping population from the cross, M507-1/Pusa 44 was used for mapping the two genes governing stripe leaf trait. As there are two genes involved in determining the stripe leaf colour in the present case, a modified strategy of two steps BSA was used for mapping the genes. These two genes were mapped onto

chromosome 5, based on the markers identified to be putatively linked to these genes through two steps BSA as described in methods section. In the present research, a set of 157 F_2 individuals from the cross, M507-1/Pusa 44 was used to map the genes tentatively named as *stp1* and *stp2*, respectively. The hyper variable SSR marker, 'HvSSR05-02' found to be closely linked with *stp1* gene mapped with a map distance of 21.7 cM on chromosome 5, while the markers RM153a and RM153b were found to be linked to second gene, *stp2* mapped at a distance of 8.6 cM and 11.9 cM, respectively on chromosome 5. In an earlier study, nearly 35,000 individuals of *wsl1*/Nanjing 11 in the F_2 population was used to fine-map white striped leaf mutant gene (*wsl1*) delimiting it to a 486.5 kb interval in the telomere region of chromosome 9. However, the recombination frequency in the telomere region of the chromosome was low during meiosis, which made the fine mapping of *wsl1* very difficult. Ye et al., (2016) fine mapped white striped leaf mutant gene (*WSL12*) between two markers, L-22 and L-17, within a 31-kb physical interval on chromosome 12 using F_2 population of *japonica* rice variety, Nipponbare and *indica* variety 93-11.

The stripe leaf-color mutant phenotype in rice can be a useful morphological marker for efficient identification and elimination of false hybrids in commercial hybrid rice production (Su et al., 2012). Mutant develops white stripe on rice leaf showing the abnormal chloroplast and cell structure in the regions with white stripe, while it was normal in the green portion of the striped leaves. This may be due to the reduction in the chlorophyll and carotenoid content in the mutant, M507-1 as compared to the normal green leaved wild type, Nagina 22. Two mutant genes tentatively named as, *stp1* and *stp2* governing the striped leaf mutations in the EMS induced mutant, M507-1 have been mapped onto chromosome 5 using SSR markers. Further detailed analysis of the mutation will help in fine mapping these genes and help elucidate the basis of stripe leaf colour mutation in the mutant, M507-1.

6 SUMMARY AND CONCLUSION

Mutation is one of the basic evolutionary processes which create useful heritable genetic variations in all living organisms including crop plants. Therefore, mutation breeding has been a powerful tool which provides the advantage of improving a specific defect in elite cultivars, without losing its agronomic and quality characteristics. It involves the creation of genetic variations through various physical and chemical mutagens, which have been employed for improving traits in crops such as rice, wheat, maize, etc. Globally, mutation breeding has been successfully used in rice, which has led to the development and release of more than 400 mutant rice varieties. Among the induced mutations for various traits in rice, more than 70 chlorophyll deficient mutants which affect leaf colour have been identified in rice which are commonly known as leaf colour mutants. Leaf color mutants are valuable genetic resources, which can provide the basic understanding on the chloroplast development and chlorophyll biosynthesis in rice. A popular upland drought, heat tolerant rice variety, Nagina 22 (N22), with a high CO_2 compensation was subjected to induced mutation by treating the seeds with ethyl methane sulphonate (EMS), from which a stable striped leaf mutant genotype M507-1, with white stripes along the leaves was obtained. The wild type Nagina 22 has a normal green leaf canopy. EMS is a chemical mutagen, which causes alkylation of bases resulting in point mutations especially G/C to T/A transitions and A/T to G/C transitions. EMS induced leaf colour mutants exhibiting variation in leaf colour such as light green, yellow, albino, purple, spots, zebra, altered shades of green, and reversible whitening have been reported in rice.

Nagina 22 possesses complete green leaf and the mutant, M507-1 exhibits a typical stripe leaf colour with white stripe leaf phenotype. This mutant has not been morphologically well characterized other than the observation on stripe leaf and there is no information on the nature of inheritance and gene(s) governing the stripe leaf trait in rice. Therefore, the present investigation entitled "**Genetic analysis and molecular mapping of *stripe* mutant in rice"** was formulated with following objectives:

1. To elucidate the inheritance of *stripe* mutation derived through the EMS induced mutagenesis.
2. Molecular mapping of the *stripe* mutant trait in rice.

In order to understand the inheritance and mapping of gene(s) governing stripe leaf trait, F_2 mapping populations from two different crosses namely, M507-1/ Nagina 22 and M507-1/Pusa 44 were developed and screened for leaf colour phenotype to ascertain the inheritance of the stripe leaf mutant trait. The parental lines of these populations M507-1, Nagina 22 and Pusa 44 were assessed for variation in photosynthetic pigments and leaf anatomy. To enable molecular mapping of the trait, polymorphic markers were identified between M507-1 and Pusa 44, which were then used for identification of putatively linked markers for the gene(s) through a modified Bulked Segregant Analysis (BSA) approach. The putatively linked markers were then used for genotyping the individuals of the F_2 populations for estimating linkage.

The major findings of the present study are summarized below:

Genetics of stripe leaf colour trait in M507-1

- A total of 333 plants of the F_2 populations from the cross, M507-1/Pusa 44 was assessed for the segregation of leaf colour. All the 333 plants were classified into two categories namely 281 plants possessing completely green (CG) and 52 plants exhibiting stripe (S) leaves, on the basis of leaf colour phenotype. The χ^2 test for goodness of fit showed that the observed data fitted well for the segregation ratio of 13:3 with *p* value of 0.1406, which showed that there are two genes governing stripe leaf colour which act in inhibitory epistatic manner.
- The segregation analysis of 369 plants of the F_2 populations from another cross, M507-1/Nagina 22, showed that there were 309 plants with completely green leaves while 60 plants showed striped leaves. The segregation ratio in this cross also showed a very good fit to 13:3 ratio (p=0.0017), confirming that the stripe leaf trait in the mutant, M507-1 is controlled by two genes interacting in inhibitory epistatic manner.
- Overall, the inheritance analysis of two F_2 populations from two different crosses namely M507-1/Pusa 44 and M507-1/Nagina 22, respectively proved that the

stripe leaf mutant trait in M507-1 is governed by two genes interacting in inhibitory epistatic manner.

Molecular mapping of gene(s) governing stripe leaf mutant trait in M507-1

- A total of 1114 SSR markers including 2 gene based/gene linked markers to stripe leaf mutation reported earlier were screened for parental polymorphism between stripe leaf mutant, M507-1 and the normal green coloured genotypes, Pusa 44 and Nagina 22, out of which 216 markers were found polymorphism between M507-1 and Pusa 44, while as many as 589 markers were found monomorphic between M507-1 and its wild type parent, Nagina 22.
- Modified Bulked Segregant Analysis (BSA) was used in which, the hyper variable SSR marker, HvSSR05-02 was found to be putatively linked with one of the gene governing stripe leaf mutant in BSA-I. Linkage analysis with the genotypic data of HvSSR05-02 showed that the first gene, tentatively named as *Stp1* was mapped to HvSSR05-02 at a distance of 21.7 cM with a very high LOD score of 8.79.
- The second BSA (BSA-II) was carried out by constituting contrasting bulks from the individuals homozygous for the mutant allele with HvSSR05-02. The SSR markers, RM153a, RM153b and RGNMS2013 could clearly differentiate between the contrasting bulks showing that these were putatively linked to the second gene governing stripe leaf mutant trait. Genotyping with SSR markers, RM153a, RM153b and RGNMS2013 showed that the second gene, tentatively named as *Stp2* was linked to two markers, RM153a and RM153b at map distance of 8.6 cM with a LOD score of 10.03 and 11.9 cM with a LOD score of 7.24, respectively.

The availability of the whole genome reference sequence of rice genome makes it easy to locate the exact physical location of the markers and the marker HvSSR05-02 was located at 0.15 Mb while the SSR markers RM153a, 153b and were located at 0.2 Mb on Chromosome 5. There seems to be a gene cluster with as many as two genes governing leaf coloration in rice on Chromosome 5. This physical position of the two genes *Stp1* and *Stp2* on Chromosome 5 is only 0.1-0.2 Mb away which is intriguing considering the fact that the marker HvSSR05-02 does not show any linkage with RM153a and RM153b in the linkage analysis. Across the rice genome, the relationship between physical distance and recombination frequency shows that for every cM there is an average physical distance of 250 kb. Since the

markers linked to the genes are very close to each other in terms of physical location, there is a need for further in-depth study to resolve the issue of linkage in this case. Further validation of the linked markers in the segregating populations in the crosses involving the mutant, M507-1 would help in answering the above question. Notwithstanding this issue, the mapping of these gene(s) would be helpful in marker assisted introgression of stripe leaf mutation to develop isogenic lines, which may help in understanding the basic mechanism involved in chloroplast development and chlorophyll biosynthesis in rice.

ABSTRACT

Rice is the major staple crop serving as major source of nutrition for more than half of the world's population and more than 90% of the global rice produce is consumed in Asia. Among the food crops, rice serves as model organism for geneticists due to the vast array of genetic and genomic resources. Induced mutations has helped in creating novel variation which has helped in understanding the genetic nature of different traits enabling its effective manipulation for rice improvement. Among the mutagenic agents, the chemical mutagen such as Ethyl Methane Sulphonate (EMS) has been widely used for creating point mutation in rice. Mutations in leaf colour help us gain insight into the mechanism of chlorophyll biosynthesis, chloroplast development and its function in plants. Therefore, the present research entitled "Genetic analysis and molecular mapping of *stripe* mutant in rice" carried out to understand the inheritance of stripe leaf trait and molecular mapping of leaf colour controlling gene(s) in the stripe leaf mutant, M507-1. A stripe leaf mutant 'M507-1' was derived through EMS-induced mutagenesis in an upland rice variety, Nagina 22, a popular drought and heat tolerant rice variety. The total chlorophyll, chlorophyll a, chlorophyll b and carotenoid in the leaf was lower in the stripe leaf mutant, M507-1, as compared to the normal green leaved wild type from seedling to maturity. The stripe leaf mutant also showed variation in the anatomy of the leaves as compared to wild plants. Analysis of the segregation for leaf colour phenotype in the F_2 population derived from two different crosses namely, M507-1/Pusa 44 and M507-1/Nagina 22 revealed that the complete green leaf colour and stripe leaf phenotype segregated in 13:3 ratio in both the crosses, indicating that the stripe leaf colour followed inhibitory epitasis. A total of 1114 SSR markers were screened for parental polymorphism between stripe leaf mutant, M507-1 and the normal green coloured genotypes, Pusa 44 and Nagina 22, out of which 216 markers were found polymorphism between M507-1 and Pusa 44, while as many as 589 markers were found monomorphic between M507-1 and its wild type parent, Nagina 22. A modified two-step Bulked Segregant Analyses (BSA) helped in identifying the marker, HvSSR05-02 linked to one of the gene, tentatively named as *Stp1*, which was mapped at a distance of 21.7 cM (LOD score - 8.79), while two SSR markers, RM153a and RM153b were found to be linked to the second gene, tentatively named as *Stp2* at map distance of 8.6 cM (LOD score - 10.03) and 11.9 cM (LOD score - 7.24), respectively, both on chromosome 5. Further in-depth analysis will help in fine mapping and identification of candidate gene(s) governing stripe leaf mutation in the mutant, M507-1, which will help in gaining insight into the basic mechanism involved in chloroplast development and chlorophyll biosynthesis in rice.

सारांश

चावल एक मुख्य खाद्यान्न फसल है, जिसका मुख्य पोषक स्रोत के रूप में विश्व की आधी जनसंख्या के द्वारा और एशिया में कुल उत्पादन का 90% से भी अधिक उपभोग किया जाता है। सभी खाद्यान्न फसलों में चावल में बहुत विस्तृत आनुवंशिक और जीनोमिक संसाधन भाग होने के कारण आनुवंशिकविज्ञ चावल को एक प्रतिदर्श जीव के रूप में उपयोग करते हैं। प्रेरित उत्परिवर्तन के द्वारा नयी विभिन्नताएं उत्पन्न करने में सहायक होते हैं, जो विभिन्न लक्षणों की आनुवंशिक प्रकृति को समझने में सहायता करते हैं, जिससे चावल विकास के लिए इसमें प्रभावी परिवर्तन किये जा सकें। विभिन्न उत्परिवर्तनजन में से रासायनिक उत्परिवर्तनजन जैसे एथिल मीथेन सल्फोनेट (ई.एम.एस.) चावल में बिंदु उत्परिवर्तन उत्पन्न करने के लिए बड़े स्तर पर उपयोग किया जाता है। पौधों के पत्ती रंग में उत्परिवर्तन से पर्णहरित के जैव संश्लेषण की क्रियाविधि, हरितलवक के विकास और इसकी कार्यिकी को समझने के लिए एक नई दिशा प्र मिलती है। इसलिए वर्तमान अनुसंधान "चावल में पट्टीधारी उत्परिवर्ती का आनुवंशिक विश्लेषण और आण्विक मापन" पट्टीधारी उत्परिवर्ती "एम 507-1" में पत्ती रंग को नियंत्रित करने वाले जीन का आण्विक मापन और पट्टीधारी लक्षण की आनुवंशिकी को समझने के लिए किया गया है। पट्टीधारी उत्परिवर्ती "एम 507-1" चावल किस्म नगीना 22 जो एक लोकप्रिय सूखा और ताप सहनशील किस्म, में ई.एम.एस. के द्वारा उत्परिवर्तन करके विकसित की गई है। एम 507-1 में कुल पर्णहरित, पर्णहरित ए, पर्णहरित बी और कैरोटेनॉयड्स की मात्रा जंगली पादप प्रकार से कम पायी गयी। पट्टीधारी उत्परिवर्ती जंगली पादप से भिन्न प्रकार की पत्ती संरचना भी दिखता है । दो भिन्न संकरण एम 507-1 / पूसा 44 और एम 507-1/ नगीना 22 से उत्पन्न की गयी द्वितीय पीढी के पौधों में पत्ती रंग लक्षण के लिए पृथक्करण का विश्लेषण किया गया, जो इन दोनों संकारणों में पर्णहरित पत्ती रंग और पट्टीधारी रंग लक्षण को 13:3 के अनुपात में पृथक करता है और यह भी दर्शाता है की पत्ती धारी रंग प्रथी रोधमातक एपीस्टेसिस का पालन करता हैं। कुल 1114 एस. एस. आर. चिन्हकों का उपयोग करके पट्टीधारी रंग उत्परिवर्ती, एम 507-1 और पर्णहरित पत्ती रंग के जननप्ररूपं, पूसा 44 और नगीना 22 के बीच जनक बहुरुपिता का अध्यनन किया गया, जिसमे से 216 चिन्हकों ने एम 507-1 और पूसा 44 के बीच बहुरुपिता दर्शायी लेकिन अधिकांशतः 589 चिन्हकों ने 507-1 और नगीना 22 के बीच एकरुपिता दर्शायी। इस अनुसंधान में एक परिवर्तित दो-पद वाली पुंज पृथक्करण विश्लेषण (बी. एस.ए.) की सहायता से एक जीन (अस्थायी नाम - *एस.टी.पी.1*) से जुड़े हुए चिन्हक "एच.वी.एस.एस.आर. 05-02" की पहचान की गयी, जो जीन से 21.5 cM (एल ओ डी अंक - 8.79) पर चित्रित किया गया, जबकी दो एस.एस.आर. "आर एम 153ए" और "आर एम 153बी के दूसरे जीन (अस्थायी नाम - *एस.टी.पी.2*) जुड़ा पाया गया और क्रोमोसोम 5 पर जीन से क्रमशः 8.6 cM (एल ओ डी अंक- 10.3) और 11.9 cM (एल ओ डी अंक - 7.24) दूरी पर चित्रित किया गया है। आगे गहन अध्ययन और विश्लेषण की सहायता से उत्परिवर्ती "एम 507-1" में पत्ती धारी पत्ती उत्परिवर्तन को नियंत्रित करने वाले विशिष्ट जीन को पहचाना और चित्रित किया जायेगा, जिससे चावल में पर्णहरित जैवसंश्लेषण और हरित लवक विकास में सम्मिलित मुख्य क्रियाविधि को देखा जा सकेगा ।

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ANNEXURE

Appendix I

Materials and Reagents

- Rice leaf samples: Leaves from both wild type parents (Pusa 44, Nagina 22) and mutant type parent (M507-1) at the maturity period usually fixed and sectioned well, at later stages the endosperm becomes progressively dry and hard, causing the sections to crumble and tear while sectioning on the microtome
- Paraplast Plus (Pelco, catalog number: 18393; Sherwood Medical, catalog number: 8889-502005; or Sigma-Aldrich, catalog number: P3683)
- Glacial acetic acid (Sigma-Aldrich, catalog number:A6283)
- Formaldehyde solution (histological grade, 37 wt% in H_2O) (Sigma-Aldrich, catalog number: 533998)
- Ethanol (96% and absolute)
- Tert-butanol (TBA) (Sigma-Aldrich, catalog number: B85927)
- Pararosaniline hydrochloride (Sigma-Aldrich, catalog number: P3750)
- Potassium metabisulfite ($K_2S_2O_5$) (Sigma-Aldrich, catalog number: 31268)
- Xylene (Xylenes, histological grade) (Sigma-Aldrich, catalog number: 534056)
- 5 M HCl (Merck-Millipore, catalog number: 1099110001)
- Decolorizing charcoal (BDH, GB) (Sigma-Aldrich, catalog number: 161551)
- DPX mounting medium (Sigma-Aldrich, catalog number: 06522)
- Formaldehyde–acetic acid –ethanol (FAA) (Recipe A)
- Dehydrating solutions (Recipe B)
- Schiff reagent (Recipe C)

Recipe A - FAA (200 ml)

- 100 ml 95% ethanol
- 70 ml dH_2O
- 20 ml 37% formaldehyde solution
- 10 ml glacial acetic acid
- Store at 4°C

Recipe B - Dehydrating solutions (for 100 ml each)

- 10 ml TBA, 40 ml 95% ethanol, 50 ml dH_2O

- 20 ml TBA, 50 ml 95% ethanol, 30 ml dH_2O
- 35 ml TBA, 50 ml 95% ethanol, 15 ml dH_2O
- 55 ml TBA, 45 ml 95% ethanol
- 75 ml TBA, 25 ml 95% ethanol
- 100 ml TBA

Recipe C - Schiff reagent (Feulgen stain)

800 ml of dH_2O boiled. Added 4 g of pararosaniline hydrochloride and dissolved while mixing. Cool to 50°C. Filter the solution through 2 glass fiber filters using vacuum. 120 ml 1M HCl and 12 g $K_2S_2O_5$ added. The solution leaved overnight in the dark at room temperature. Added 2 to 4 g decolorizing charcoal in the solution and mixed. Filter the solution through 2 glass fiber filters using vacuum into a dry bottle (the stain must be clear and colorless). Schiff reagent was store in the refrigerator at 4°C for up to 1 year (used until precipitate starts forming in the reagent).

Equipments and other accessories for fixation of leaf samples

- Glass vials (volume ~20 ml)
- Glass fiber filters (Microfibre filters, Whatman GF/C)
- Scalpels, razor blades
- Metal forceps (use dedicated forceps for wax work, they are difficult to clean)
- Disposable plastic Pasteur pipettes
- Vacuum desiccator (Bel-Art, model: 420100000)
- Staining dishes (for melting Paraplast Plus) (Electron Microscopy Sciences, catalog number: 70312-21)
- Hot plate (set on low –around 68°C), with aluminum foil tent to keep the heat inside
- Alcohol burner
- Plastic or metal molds Disposable Base Mold (Pelco, catalog number: 27147) Tissue-Tek Base Mold (Sakura, catalog number: 4123)
- Tissue embedding cassette bases (Pelco, catalog number: 27168-1) or embedding rings (Pelco, catalog number: 27169-1) or Tissue-Tek Embedding Rings (Sakura, catalog number: 4151)

- Small beaker with molten Paraplast Plus (dedicated for wax work)
- Tray with a thin layer of cold water (cool it by adding ice)
- Ice bucket filled with ice
- Flat ice packet (frozen cooling pack) or brass plate (optional)
- Rotary microtome
- Disposable microtome blades
- 18 Small brush
- Forceps with sharp ends
- Single-edged razorblade
- Dark cardboard plate
- Stereomicroscope
- Coplin jars (Electron Microscopy Sciences, catalog number: 70316-02)

Appendix II

Equipments and other accessories for casting wax blocks

- Hot plate (set on low –around 68°C), with aluminum foil tent to kept the heat inside
- Metal forceps (used dedicated forceps for wax work, they were difficult to clean)
- Alcohol burner
- Plastic or metal molds
- Embedding rings or embedding cassette bases
- A small beaker with molten Paraplast Plus (also has to be dedicated for wax work)
- A tray with a thin layer of cold water (cooled it by adding ice)
- An ice buckets filled with ice
- Flat ice packet (frozen cooling pack) or brass plate (optional)

Appendix III

Equipments and other accessories for sectioning:

- Rotary microtome
- Disposable microtome blades
- A small brush
- A forceps with sharp ends
- A single-edged razor blade
- Dark cardboard plate (for putting the sections on and observing them)
- A stereomicroscope (optional but recommended)
- Hot plate set on 40°C
- A small jar with distilled water
- A plastic pipette
- Cleaned objective microscope slides, frosted on one edge
- A pencil

Appendix IV – Chemical reagents for extraction of genomic DNA

Cetyl-trimethyl ammonium bromide (CTAB): CTAB (2 g) as detergent used for 100 ml DNA extraction buffer to break cell wall and remove impurities from leaf samples.

Trizma-base (1 M, pH 8.0): Trizma base 30.28 g (MW = 121.1 g) was dissolved in 200 ml of distilled water. The pH was adjusted to 8.0 with concentrated HCI. The final volume of the solution was made up to 250 ml with distilled water before autoclaving. The solution was then stored at room temperature (25°C) after autoclaving. Trizma-base ensures the pH of the buffer solution.

Ethylene diamine tetra acetate (EDTA, 0.5 M, pH 8.0): EDTA (46.53 g, MW = 372.2 g) was added to 200 ml of distilled water. It was stirred vigorously with a magnetic stirrer and maintained at pH 8.0 using NaOH pallets and concentrated NaOH solution. The final volume of the solution was made up to 250 ml with distilled water before autoclaving. The solution was stored at room temperature (25°C) before final adjustment of the pH after autoclaving. EDTA performs as chelating agent that help to remove several kinds of divalent metal cations such as Mg^{2+} and Ca^{2+} that act as co-factor for the majority of DNases which degrade DNA.

Sodium Chloride (NaCl, 5 M): NaCl (73.05 g, MW = 58.44 g) was dissolved in 200 ml and stirred vigorously with a magnetic stirrer. The final volume was adjusted to 250 ml with distilled water before autoclaving. The solution was stored at room temperature (25°C) after autoclaving. NaCl is a salt that increases the solubility of DNA in the buffer solution and also increases the osmotic ability of the buffer and hence facilitates the process of cell lysis.

Chloroform: Isoamyl alcohol mixture: This is used in the proportion of 24:1 to remove proteins by denaturation and after this they aggregate in the intermittent phase along with cell debris.

RNase (10 mg/ml): RNase (~100 mg) was dissolved in 10 ml of sterile distilled water in a sterile 15 ml centrifuge tube. It was dispensed into sterile 1.5 ml micro-centrifuge tubes and stored at –20°C. The RNA is removed by RNase treatment at 37°C.

Ethanol (70%): The pellet is washed with 70% ethanol for removing any salts retained after precipitation.

Appendix V – Preparation of the DNA Extraction buffer

Composition of DNA extraction buffer (for 100 ml)

Chemicals	Final Concentration	Working volume
1 M Tris HCl (pH 8.0)	100 mM	10 ml
0.5 M EDTA (pH 8.0)	20 mM	4 ml
5 M NaCl	1.4 M	28 ml
CTAB	2%	2 g
Double distilled H_2O	-	58 ml
β–Mercaptoethanol*	0.1%	100 µl

* β-Mercaptoethanol (100 µl) was added just prior to place DNA extraction buffer in water bath for incubation at 65°C.

Appendix VI – List of polymorphic molecular marker, their linkage distance and sequences

Sl.	Marker	Chr	PP	Forward primer	Reverse primer	Reference
1.	RM499	1	0.39	TACCAAACACCAACACTGCG	ACCTGCAGTATCCAAGTGTACG	Temnykh et al., 2001
2.	RM8086	1	1.66	GAAGTCGTAGCTATAGCTAG	TAAATGTACGTACATATATATACATA	McCouch et al., 2002
3.	RM1321	1	3.8	TTAATTTGCCATTCCCAAC	CTAGTGCCTCGGTGAAATAC	McCouch et al., 2002
4.	RM1167	1	4.23	GAACATAAACCATGCGGGAG	AGCTAGTGGCAAAAGTGTGC	McCouch et al., 2002
5.	HVSSR 01-07	1	4.35	ACGGGAGATTCACACATAGA	ATTATGGTATGCTTTGCCAG	Singh , 2009
6.	RM220	1	4.42	GGAAGGTAACTGTTTCCAAC	GAAATGCTTCCCACATGTCT	Chen et al., 1997
7.	RGNMS3235	1	7.52	AATTGTAGATTTGTTGCATCG	AAAGAAGATTGCTCTGTGATTT	Parida et al., 2009
8.	RM14	1	8.67	CCGAGGAGAGGAGTTCGAC	GTGCCAATTTCCTCGAAAAA	Panaud et al., 1996
9.	RM572	1	9.87	CGGTTAATGTCATCTGATTGG	TTCGAGATCCAAGACTGACC	Temnykh et al., 2001
10.	RM35	1	12.17	TGGTTAATCGATCGGTCGCC	CGACGGCAGATATACACGG	Chen et al., 1997
11.	RM493	1	12.28	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG	Temnykh et al., 2001
12.	RM10835	1	13.55	GGCACTTTGTCTATTTCCTTCC	GGCAGTACAATCAATCCATACC	Sasaki et al.,, 2005
13.	RM10852	1	13.97	GAATTTCTAGGCCATGAGAGC	AACGGAGGGAGTATATGTTAGCC	Sasaki et al.,, 2005
14.	HVSSR 01-44	1	14.23	TGAGTGAGACTTGACAGTGC	AGTTAACACCAATGCTGACC	Singh , 2009
15.	RM449	1	15.12	TTGGGAGGTGTTGATAAGGC	ACCACCAGCGTCTCTCTCTC	Temnykh et al., 2001

16.	RM6711	1	16.11	TAGTGATAGGGGTGGTGTGG	TTACAAGCATGGGAGTTGGG	McCouch et al., 2002
17.	RM129	1	19.01	TCTCTCCGGAGCCAAGGCGAGG	CGAGCCACGACGCGATGTACCC	Temnykh et al., 2000
18.	RM9	1	23.33	GGTGCCATTGTCGTCCTC	ACGGCCCTCATCACCTTC	Panaud et al., 1996
19.	RM306	1	23.93	CAAGGTCAAGAATGCAATGG	GCCACTTTAATCATTGCATC	Temnykh et al., 2000
20.	RM5	1	23.97	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG	Panaud et al., 1996
21.	RM3475	1	26.04	GTCGGTTTGCCTAGTTGAGC	TTCCTCGGTGTATGGGTCTC	McCouch et al., 2002
22.	RM246	1	27.34	GAGCTCCATCAGCCATTCAG	CTGAGTGCTGCTGCGACT	Chen et al., 1997
23.	RM1152	1	30.09	GCCTTTGTCCTTCAGTAGGC	AGAGCGCCTGGGTATAATTG	McCouch et al., 2002
24.	RGNMS348	1	32.84	TTAGGATTATGGATTTGTAGGAAA	GCCATGAAACATAAACATAAACTA	Parida et al., 2009
25.	RM212	1	33.05	CCACTTTCAGCTACTACCAG	CACCCATTTGTCTCTCATTATG	Chen et al., 1997
26.	RM5501	1	34.54	GCGCTTCTACTTCCACAAGG	GGTTGGCGTACGTAGAGAGG	McCouch et al., 2002
27.	RM8084	1	34.9	CATGACAACATTGTTCTGAA	CCTGTGATCAAGTCCTGTAG	McCouch et al., 2002
28.	RM11841	1	35.64	AACTTGCACACCGCACAACTCC	TGCTGTATGGCCATGTGGATGC	Sasaki et al.,, 2005
29.	RM5389	1	35.73	TCTTGCATGAGAGCCAACAC	GCTATTGCGCGAGATTATCC	McCouch et al., 2002
30.	RM3825	1	36.47	AAAGCCCCCAAAAGCAGTAC	GTGAAACTCTGGGGTGTTCG	McCouch et al., 2002
31.	RM11918	1	37.33	AGAAGAGGTCAAGACAGGAAAGTAGG	TGCTGGATTGAGACACATTTGC	Sasaki et al.,, 2005
32.	RM1339	1	38.19	ATCAAAGCATGTAAACCAGC	CGTAAGATCTCCCTACCACC	McCouch et al., 2002

33.	RM6696	1	38.22	TGGTCCACACGTGATGAGTC	GAAGATAGGGGAGTGGGGAG	McCouch et al., 2002
34.	RM431	1	38.89	TCCTGCGAACTGAAGAGTTG	AGAGCAAAACCCTGGTTCAC	Temnykh et al., 2001
35.	RM12055	1	39.63	GGCCAAATGGACTAAGAAGAAGG	CCCGATGCTTGAAGATATAGTGG	Sasaki et al.,, 2005
36.	RM3482	1	39.71	TTGTTGTCAAGCTACGGTGG	CTGCTTCGTGATGTTGTTGG	McCouch et al., 2002
37.	RM3523	1	39.82	TCGATCGCTCGCTCTCTC	GGATATGGAGCGCAAGAGAG	McCouch et al., 2002
38.	RM12204	1	41.81	CCTTCCTAATACCCTGTCATCTGC	GCCCTTTCTTTCACTTGATTGG	Sasaki et al.,, 2005
39.	RM12230	1	42.38	CACCTGTTGTGAATCTGTGATCG	CGGTGGAGCTTGTTATACTACTATGG	Sasaki et al.,, 2005
40.	RM1067	1	42.92	CGATGGAGAGAGAATGTCTAGC	TAATACGCAAGGCAGAAGGG	McCouch et al., 2002
41.	HVSSR 02-01	2	0.12	AAGAGATGAGAAGAGCAATGA	CAACTTAGAGGAAGAAGGAGG	Singh, 2009
42.	HVSSR 02-10	2	4.29	GAACTGGAGTTGCAGATTTC	GTTCATGATGCTTGTTGCTA	Singh, 2009
43.	RM6247	2	5.8	CGCTCTTGTCTTTACTCCCG	GCTGCTGCTGCTTCTTTTTC	McCouch et al., 2002
44.	RM12811	2	7.77	ACAGTACACTAGGACATGGTTACG	GTCTAATTGCAGAATGCAGACC	Sasaki et al.,, 2005
45.	RM12812	2	7.77	GTCCCAGTTGACGGTCAGGTACG	GTCCACAGGGTCGTCATGTGC	Sasaki et al.,, 2005
46.	RM53	2	7.98	ACGTCTCGACGCATCAATGG	CACAAGAACTTCCTCGGTAC	Chen et al., 1997
47.	RM71	2	8.76	CTAGAGGCGAAAACGAGATG	GGGTGGGCGAGGTAATAATG	Temnykh et al., 2000
48.	RM5699	2	8.98	ATCGTTTCGCATATGTTT	ATCGGTAAAAGATGAGCC	McCouch et al., 2002
49.	RM324	2	11.39	CTGATTCCACACACTTGTGC	GATTCCACGTCAGGATCTTC	Temnykh et al., 2000

50.	RM301	2	12.21	TTACTCTTTGTGTGTGTGTGAG	CTACGACACGTCATAGATGACC	Temnykh et al., 2000
51.	RM13088	2	13.06	GTGCAACGACTTCATTCAACC	GATGGTTTCTTACGGTCTCATGG	Sasaki et al.,, 2005
52.	RM13150	2	14.92	CAAAGTCCGAAGGAATTGTACTGC	GATGCGGGAGATTATGCATGG	Sasaki et al.,, 2005
53.	RM13263	2	18.09	AAGATTGCACACTGGTGTTCTCC	AGAAGAGCCGGTCTTTGTCTCC	Sasaki et al.,, 2005
54.	RM6318	2	24.42	TGCTGCTTCTGTCCAGTGAG	GGATCATAACAAGTGCCTCG	McCouch et al., 2002
55.	RM221	2	27.61	ACATGTCAGCATGCCACATC	TGCAAGAATCTGACCCGG	Chen et al., 1997
56.	RM5460	2	33.77	AAGAGAACAAGCCATGGTGC	GCCTTTTCTTGCCTTTGGAC	McCouch et al., 2002
57.	RM5916	2	34.05	GCTATAAGAATCGTATTAAG	TACTGCTATTAAAGTCAGAA	McCouch et al., 2002
58.	RM3789	2	34.68	ATTAAGGGCAGGGGCATATC	CATTGACTGGTGTGGTCAGG	McCouch et al., 2002
59.	RM138	2	35.36	AGCGCAACAACCAATCCATCCG	AAGAAGCTGCCTTTGACGCTATGG	McCouch et al., 2002
60.	RM535	2	35.77	ACTACATACACGGCCCTTGC	CTACGTGGACACCGTCACAC	Temnykh et al., 2001
61.	RM22	3	1.52	GGTTTGGGAGCCCATAATCT	CTGGGCTTCTTTCACTCGTC	Temnykh et al., 2001
62.	RM489	3	4.33	ACTTGAGACGATCGGACACC	TCACCCATGGATGTTGTCAG	Temnykh et al., 2001
63.	RM3864	3	5.86	AGTCAACCTTGGGGGTAAGG	AGATACTGCCCGTGTCATCC	McCouch et al., 2002
64.	RM3716	3	6.84	GTCGTTCGGTTGACTCGTTG	CACACATATATACCCCCCCC	McCouch et al., 2002
65.	RM3766	3	6.93	TTATAGAGCCAACACAACGG	ATCGATCTCTCTCCTGGAAA	McCouch et al., 2002
66.	RM14639	3	6.97	ATAAGAGGACTACGCCAAACAACACC	ATGGCTGGAATCCATTCTTTGG	Sasaki et al.,, 2005

67.	RM3461	3	8.35	AAAGTCTCCCTGTTGTAGCC	CATGAACGTAAAGCAAACG	McCouch et al., 2002
68.	RM5803	3	9.32	GGAGGGGAAGGATAAGGAGG	TCTCTTCCACCAACTCCACC	McCouch et al., 2002
69.	RM1284	3	10.62	ACACTCCCCTTTTGTAAAGC	CTAGAACTACGGCATTTTCG	McCouch et al., 2002
70.	RM2794	3	11.33	TAGCTTTTTCGTCAGTGTTA	CATTAGCCGTTACATACTTG	McCouch et al., 2002
71.	RM7	3	11.85	TTCGCCATGAAGTCTCTCG	CCTCCCATCATTTCGTTGTT	Panaud et al., 1996
72.	RM15283	3	18.75	GCTACAAATAGCTGCAAACTGC	TTGGACTAGCCTTTGACTGAGG	Sasaki et al., et al. 2005
73.	RGNMS1373	3	21.21	CTCGACCAAACAGACCAGTA	CTTAGGAGAATCTGGCGGTG	Parida et al., 2009
74.	RM3646	3	21.99	ACTAGAGCACCCTCGCTGAG	CTCAGCCACCCCATCAAC	McCouch et al., 2002
75.	RGNMS1140	3	27.23	ATATGCGCTTCCTCTTCGTT	AAATTCACAAACTCTCGCTATT	Parida et al., 2009
76.	RM186	3	28.8	TCCTCCATCTCCTCCGCTCCCG	GGGCGTGGTGGCCTTCTTCGTC	Temnykh et al., 2000
77.	RM520	3	30.91	AGGAGCAAGAAAAGTTCCCC	GCCAATGTGTGACGCAATAG	Temnykh et al., 2001
78.	RM571	3	33.16	GGAGGTGAAAGCGAATCATG	CCTGCTGCTCTTTCATCAGC	Temnykh et al., 2001
79.	HVSSR 03-93	3	34.56	GATCTAGGCACAAGGCATAC	TTTCCTTCTTCTGTAAACGC	Singh, 2009
80.	RM2187	3	35.61	GTCATTTGAAGTAAATCCGT	GGTCTACTTGCGAAATAAGT	McCouch et al., 2002
81.	RM55	3	36.41	CCGTCGCCGTAGTAGAGAAG	TCCCGGTTATTTTAAGGCG	Chen et al., 1997
82.	RGNMS1135	3	36.35	ATGGTCATAAGAGCCTAGAGAC	TCAATGCTTTGTGACTTATAGC	Parida et al., 2009

83.	RM16281	4	0.39	CCATTCTGTTGTTCTCACACTTGC	TGCGACATCAGAGTAGTGTTGTAAGG	Sasaki et al.,, 2005
84.	RM16285	4	0.55	GCTCAAGATGGAATAACGATGG	GAGCTTTCATCGGATTCTCG	Sasaki et al.,, 2005
85.	RM16303	4	1.1	CTCACTCTGTCCACAAATACAGG	GTTGGGTGGATAGACAGTGG	Sasaki et al.,, 2005
86.	RM518	4	2.03	CTCTTCACTCACTCACCATGG	ATCCATCTGGAGCAAGCAAC	Sasaki et al.,, 2005
87.	RGNMS1454	4	4.45	AGACGCCTACCGGTGATAAT	AATAGTTGATTTGAGGTGTGGT	Parida et al., 2009
88.	RM1359	4	7.9	AACGAATTCTATTTTGCGTC	TTCTTCTCATTTCAATTCGC	McCouch et al., 2002
89.	RM16611	4	11.71	CGGAGATGCGAGAGACAGACG	GTGTCCGTTCCCACTGCAAGC	Sasaki et al.,, 2005
90.	RM401	4	13.15	TGGAACAGATAGGGTGTAAGGG	CCGTTCACAACACTATACAAGC	Temnykh et al., 2001
91.	RM307	4	13.15	GTACTACCGACCTACCGTTCAC	CTGCTATGCATGAACTGCTC	Temnykh et al., 2000
92.	RM3317	4	13.65	AGCAACCTGACAGAAGAATG	TCTCGTTGAGTTGGAAGAAG	McCouch et al., 2002
93.	HVSSR04-21	4	15.57	GTAGGAAGAGAAGAAGGGAAG	ATGACTTATGGGATGGAATGT	Singh, 2009
94.	RGNMS1507	4	15.75	TTAAGAAGATTAAGGCTGTGTTC	ACCTATAGATAACCATTGTGGG	Parida et al., 2009
95.	RM16745	4	16.63	TGAGGAGTGAGGAGAGTGATAAACC	GCATATGGTTTGGTTGATGTCC	Sasaki et al.,, 2005
96.	HVSSR04-28	4	20.23	ATGGATTTAGGCTTGTTTGA	ATACTGCGAAGGTGAAGAGA	Singh, 2009
97.	RM17389	4	20.24	AGATATCAACCGGCAGAAGG	GACTACCTCTTGCTTTGGTAGG	Sasaki et al.,, 2005
98.	RM1155	4	20.34	AGGGAGTGTGGCAACTATGC	GGGAGGAGTGAGAAGGGATC	McCouch et al., 2002
99.	RM2439	4	22.91	ATGTTTAGATTCTTAGCACT	GCTCATATCCATATAAATGT	McCouch et al., 2002

100.	RM470	4	28.09	TCCTCATCGGCTTCTTCTTC	AGAACCCGTTCTACGTCACG	Temnykh et al., 2001
101.	RM303	4	28.57	GCATGGCCAAATATTAAAGG	GGTTGGAAATAGAAGTTCGGT	Temnykh et al., 2000
102.	HVSSR04-46	4	30.49	CTAGTGGGTAGCATGAGAGG	CAAATTCAGTTCAAACTTGCT	Singh, 2009
103.	RM3276	4	30.49	TCCGTCTCGACTCTTCCATC	GATGAGACACCACGGACATG	McCouch et al., 2002
104.	HVSSR04-47	4	30.64	GGCGCGCTTATATATGTACT	CGATTGCGTGGTGTAACTAT	Singh, 2009
105.	RM3687	4	31.29	CTCCTGAGAAGTGGGGACTG	AGTCCTCCATGCATGTGACC	McCouch et al., 2002
106.	RM1113	4	34.09	GGGCGCATGTGTATTTCTTC	TGGGGAAAAACCACAAGCC	McCouch et al., 2002
107.	RM567	4	34.53	ATCAGGGAAATCCTGAAGGG	GGAAGGAGCAATCACCACTG	Temnykh et al., 2001
108.	RM280	4	34.98	ACACGATCCACTTTGCGC	TGTGTCTTGAGCAGCCAGG	Temnykh et al., 2000
109.	RM2431	4	35.13	ATTGGAATACCTAAAAGCTA	GGATGCCTTATATGAAATAT	McCouch et al., 2002
110.	NKSSR04-11	4	30.9	CCATCAGTTGAAGGGCTCTC	CTTTTATGGCATGGGCAACT	Deshmukh et al., 2010
111.	NKSSR04-19	4	31.26	CTGGAATCACAAACCACGAC	GCTACCTCAAGCTCCACGAC	Fan et al., 2010
112.	HVSSR 05-02	5	0.15	TATTGGCCATTGATTACTCC	CATCTTACAAACTGAAACGGA	Singh, 2009
113.	RM153b	5	0.18	CCTCGAGCATCATCATCAG	TCCTCTTCTTGCTTGCTTCT	McCouch et al., 2002
114.	RM153a	5	0.19	GCCTCGAGCATCATCATCAG	ATCAACCTGCACTTGCCTGG	McCouch et al., 2002
115.	HVSSR 05-13	5	3.11	TCCTCTACAGTTGTCTGCCT	CATTCCTCTCCACTTTCTTG	Singh, 2009
116.	RM5874	5	3.53	GAAAAGATCCTGGCTCGTTG	GCATCATCGCCAGAGCTC	McCouch et al., 2002

117.	RM18048	5	5.79	GATCACTAGCAGAGGCACAAGAGG	ATGGCAAGTTAACAGCGACATCC	Sasaki et al.,, 2005
118.	RM289	5	7.81	TTCCATGGCACACAAGCC	CTGTGCACGAACTTCCAAAG	Temnykh et al., 2000
119.	HVSSR05-25	5	9.12	AGCGTGGCTACATTCACTAT	GCTTGTCTTGGAAATTGTTC	Singh, 2009
120.	RM5844	5	9.15	TGACTAACGTGGCATCCATG	GCTAGGAGCCATTGTCGAAG	McCouch et al., 2002
121.	RM18237	5	10.41	-	-	
122.	RM1127	5	15.97	CTTAGTGCCGGACCTCACTC	CCTCATCTCCCTCATCCATC	McCouch et al., 2002
123.	HVSSR05-45	5	19.82	GTGCATTTGCAACTTAAACA	GGGAGATCAAGAAGAGGTTT	Singh, 2009
124.	RM3351	5	20.69	ATGGAAGGAATGGAGGTGAG	TACCCCTACGTCGATCGATC	McCouch et al., 2002
125.	RM3663	5	21.36	CATCAACCTCCACGAACATG	CTCGGTGGTGATCCTCCTC	McCouch et al., 2002
126.	RM3575	5	21.39	CCTGGAATGATGATGGAAGG	GTTTTGCTTCCTGGAAGTGC	McCouch et al., 2002
127.	RGNMS3250	5	-	CGTTTATAGAACACCACACAAC	TTTGACCATTTGTCTTATTCAA	Parida et al., 2009
128.	RGNMS2013	5	24.95	CTCCTCTCTCTTGTTGGTGA	TTATGCTGGACTATTTGCCT	Parida et al., 2009
129.	RM19274	6	1.01	CCTGTGAATGACAACCCATGC	GTATGAGCCAGATTAGCGGTTGC	Sasaki et al.,, 2005
130.	RM2353	6	1.06	GACGTACCTAACATTTGACA	CCAAGTGATAAAAAAAGACC	McCouch et al., 2002
131.	RM19291	6	1.22	CACTTGCACGTGTCCTCTGTACG	GTGTTTCAGTTCACCTTGCATCG	Sasaki et al.,, 2005
132.	RM19297	6	1.24	ATTTGCTCCGCTTCCAAATCACC	AGCGGCCAACAGAGACAACTGG	McCouch et al., 2002
133.	RM19296	6	1.29	CTAGCTTGACGCCAAGGACACC	GCACAGACGCACACTGATCTCC	McCouch et al., 2002

134.	RM597	6	1.37	CCTGATGCACAACTGCGTAC	TCAGAGAGAGAGAGAGAGAGAGAG	Temnykh et al., 2001
135.	RM589	6	1.38	ATCATGGTCGGTGGCTTAAC	CAGGTTCCAACCAGACACTG	Temnykh et al., 2001
136.	RM204	6	3.17	GTGACTGACTTGGTCATAGGG	GCTAGCCATGCTCTCGTACC	Chen et al., 1997
137.	RGNMS2178	6	7.08	ATTAGAGCATCTCCTTCTTTGA	AAGAAGAAGAACAAGCAGAGAG	Parida et al., 2009
138.	RGNMS3603	6	9.5	GTAACTGTAAGCTCCTCATGG	CCTATCCATCTTCATCAAGTCT	Parida et al., 2009
139.	HVSSR06-56	6	25.67	AGCATTTGTGTGTGCAATAG	ATGCTTGCCTCATCAGTAGT	Singh, 2009
140.	RM528	6	26.55	GGCATCCAATTTTACCCCTC	AAATGGAGCATGGAGGTCAC	Temnykh et al., 2001
141.	RM30	6	27.25	GGTTAGGCATCGTCACGG	TCACCTCACCACACGACACG	Panaud et al., 1996
142.	RM400	6	28.43	ACACCAGGCTACCCAAACTC	CGGAGAGATCTGACATGTGG	Temnykh et al., 2001
143.	HVSSR06-75	6	30.18	CCAACAGTTTCAAGAGGAAG	CTGCTTCATGTATCCTAGCC	Singh, 2009
144.	RM176	6	30.27	CGGCTCCCGCTACGACGTCTCC	AGCGATGCGCTGGAAGAGGTGC	Temnykh et al., 2000
145.	RGNMS2220	6	30.98	TCCACACAATATTGATCATCTC	CCACGTCGTTATATTGTTACTG	Parida et al., 2009
146.	RM21264	7	7.41	CAGACGATGACGATGATGACTGC	ACAGCCTGCTTCCCTCTCTCC	Sasaki et al.,, 2005
147.	RM47	7	8.71	ACTCCACTCCACTCCCCAC	GTCAGCAGGTCGGACGTC	Chen et al., 1997
148.	RM21462	7	14.45	TCCTGCATCAACATCCAACACC	TCCGTTCTGGCAGCAAATGG	Sasaki et al.,, 2005
149.	RM182	7	14.88	GAGTGCAGTTGGCATCAAAG	TACCACCAATGAATGCCGGAG	Akagi et al., 1996
150.	RM70	7	15.76	GTGGACTTCATTTCAACTCG	GATGTATAAGATAGTCCC	Chen et al., 1997

151.	RM21606	7	17.88	TGAGAAGGGAGATGAAAGGAAGC	GGACGGAGGGAGTAATGAATTGG	Sasaki et al.,, 2005
152.	RM3691	7	19.23	GCTGATGGTCAAAGATCAGG	ATGTGTCTGCTGGCACAGAG	McCouch et al., 2002
153.	RM6965	7	24.64	TCATTTGGATCATAAGCTGG	TTGGATGAGATAACCAATGC	McCouch et al., 2002
154.	RM473	7	25.46	TATCCTCGTCTCCATCGCTC	AAGGATGTGGCGGTAGAATG	Temnykh et al., 2001
155.	RM2715	7	28.65	CTACTTTCTCCGTTTCATAA	CATTTCTCACATTCATATTG	McCouch et al., 2002
156.	RGNMS 3214	8	8.57	TGGGAACAAAGAAGATAGAGAT	TTACTATCTAGTGCTGCGAGTG	Parida et al., 2009
157.	RM22990	8	17.55	CTGGTAGCTCTTCGACCACTGC	GGAACGAATGAGTAGCATCTTAGGG	Sasaki et al.,, 2005
158.	RM6182	8	20.13	CCCGTGAAATGCGTAAAG	GATTCCATCCTAGCACCTC	McCouch et al., 2002
159.	RM23130	8	20.37	CACCTAAGAGCGAATGTATAGG	CGATCTACGATCTGATTACACC	McCouch et al., 2002
160.	RM5637	8	21.24	CAACTCCAACGACGATGAAC	TGGTGAAGTGGAGTGGAGTG	McCouch et al., 2002
161.	RM23237	8	22.32	TAAAGCATTTGGACGGTGGATGG	GAGGTGGGTGTGACCCTTGG	McCouch et al., 2002
162.	RM23309	8	23.53	CGAATCCAAACCCTAAATCTCTCC	GCGCGAGAGAGAAACAGAGAGG	McCouch et al., 2002
163.	RM23408	8	25.08	CCATCTCAACTCCTTCGTTTACTGC	TCGACTGTTTGCTTGGAATAGGC	Sasaki et al.,, 2005
164.	RM23623	8	28.14	TCGTAGGAACAATGGAACACG	TAAATCTTGGAGCCCATACTCC	McCouch et al., 2002
165.	RM433	8	28.30	TGCGCTGAACTAAACACAGC	AGACAAACCTGGCCATTCAC	Temnykh et al., 2001
166.	RM285	9	0.44	CTGTGGGCCCAATATGTCAC	GGCGGTGACATGGAGAAAG	Temnykh et al., 2000
167.	RM23741	9	2.68	AGACTCAATAGCGGTCCATTTCC	CCTTAGGATGCTAACTGCAAAGACC	Sasaki et al.,, 2005

168.	RM444	9	5.93	GCTCCACCTGCTTAAGCATC	TGAAGACCATGTTCTGCAGG	Temnykh et al., 2001
169.	RM1896	9	11.77	GGACAGGGTAAAGTGTTAGA	CCTAAGACCTATCAACTCCA	McCouch et al., 2002
170.	RM105	9	12.55	GTCGTCGACCCATCGGAGCCAC	TGGTCGAGGTGGGGATCGGGTC	Temnykh et al., 2000
171.	HVSSR09-36	9	16.45	AAGCTAACTAGGATGGTGGC	TTGGAAGAAGAGCATAGAGTC	Singh, 2009
172.	RM215	9	21.18	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG	Chen et al., 1997
173.	RM3917	9	22.48	AATGTATTAGGATAAATGCGAAG	GAACGAACGTGAATGAGAAC	McCouch et al., 2002
174.	RM245	9	27.39	ATGCCGCCAGTGAATAGC	CTGAGAATCCAATTATCTGGGG	Chen et al., 1997
175.	RM6271	10	4.79	ACCTCAAGATTCCAGCTGTC	AAGCGGAAATGCTGCAGTAG	McCouch et al., 2002
176.	RM271	10	14.49	TCAGATCTACAATTCCATCC	TCGGTGAGACCTAGAGAGCC	Temnykh et al., 2000
177.	RM4	11	0.93	TTGACGAGGTCAGCACTGAC	AGGGTGTATCCGACTCATCG	Panaud et al.,1996
178.	RM3717	11	1.17	AGCTCTACCTTTGCTGTCGG	AACTCCCTAGACCCACCTGC	McCouch et al., 2002
179.	RM2459	11	2.4	AGTTTGAAGTTTGTCTTGAA	AGTTACCAAAAGTTTAATCG	McCouch et al., 2002
180.	HVSSR11-12	11	2.48	AACCTCATCGTATCCTCCTC	ACTAGTGAGTCCCACGTGTC	Singh, 2009
181.	RM552	11	4.84	CGCAGTTGTGGATTTCAGTG	TGCTCAACGTTTGACTGTCC	Temnykh et al., 2000
182.	RGNMS3233	11	5.05	ACATCGATTTCCTCATCAAG	TTCCACCTTCAACAAGCTAC	Parida et al., 2009
183.	RM441	11	6.08	ACACCAGAGAGAGAGAGAGAGAG	TCTGCAACGGCTGATAGATG	Temnykh et al., 2001
184.	RM26281	11	6.49	AACAAATCCCACATGACGATGC	GCCCTAGTCAGTCCCTCTGTAATCC	Sasaki et al.,, 2005

185.	RGNMS3464	11	6.55	GCCTCCTCTCTATCTTCTTTCT	AAATCATTCACAAATTTCCATC	Parida et al., 2009
186.	RM26323	11	7.46	ACACGCCTTGACGAGGTGTCTCC	CGCCGTGTTCACGTTACAGAGG	Sasaki et al.,, 2005
187.	RM536	11	8.99	TCTCTCCTCTTGTTTGGCTC	ACACACCAACACGACCACAC	Temnykh et al., 2001
188.	RGNMS3458	11	9.3	CACTCGCAGCACTAGATAGTAA	TAGTAAGTATCGATCCAGAGCC	Parida et al.,2009
189.	RM27260	11	12.03	GTGCTGAATTCCTCTCGTTACGC	GATCGCCACCGAAGTACTACCC	McCouch et al., 2002
190.	RM287	11	16.77	TTCCCTGTTAAGAGAGAAATC	GTGTATTTGGTGAAAGCAAC	Temnykh et al., 2000
191.	RM209	11	17.81	ATATGAGTTGCTGTCGTGCG	CAACTTGCATCCTCCCCTCC	Chen et al., 1997
192.	RGNMS3232	11	19.76	ACTGGCTGTACCGTACTAGTTT	CTTTCTATCTTCGCTCTCAGTC	Parida et al.,2009
193.	RM26918	11	20.14	GTGGCGGTATAAGAGCGTTTGC	CTCCATGTGCACAATCCAGTTCC	McCouch et al., 2002
194.	RM26923	11	20.16	CAATTCATAACGTCGGTCCTTCC	TATTTGCATGGCCCTGTTTGC	McCouch et al., 2002
195.	RM26973	11	21.22	TGTAGGTGCGTTTATGGAATACCG	CCTCCGTCTCCTATCTCCTATCG	McCouch et al., 2002
196.	RM206	11	22.01	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG	Chen et al., 1997
197.	RM5997	11	22.71	GCGACGACGAAGAAGCTAAC	CCCATCGATAGGGTTTCCTC	McCouch et al., 2002
198.	RM27117	11	23.75	GTACAATGGATCATCCCAAACC	AGTCATGTCTAGCTTCTCCTTCG	McCouch et al., 2002
199.	RM27138	11	24.11	TTCACACGTTTGACCATTCG	GCCTTGATGTTCTGCTCTATTGG	McCouch et al., 200
200.	HvSSR11-76	11	25.33	CGACCTCCGAACACAGCC	TTCTTCTCCCAACCCTTC	Singh, 2009
201.	RM144	11	28.28	TGCCCTGGCGCAAATTTGATCC	GCTAGAGGAGATCAGATGGTAGTGCATG	Temnykh et al., 2000

202.	RM7443	11	28.43	TGCTGCGTGTTACTTTGGTG	AACCCTTCATCAGGCTACGC	McCouch et al., 2002
203.	RM27462	12	1.1	ATCCCTCTCCCTCAACCAAATCC	CCCGAGTTCTCTCCCATCTCC	Sasaki et al.,, 2005
204.	RM7619	12	4.83	CTTGGTATGTATTGGCAGCG	GAGGCAATAGGAGGGGAGAG	McCouch et al., 2002
205.	RGNMS3130	12	7.46	TTGATGTTTCCATACTTCTTGA	TAAATGCTGAGCTAAATCAGTG	Parida et al.,2009
206.	HVSSR12-19	12	9.27	AGGCCTTCTTTAATTGATCC	CGATCTATCCATGAGCAAAT	Singh, 2009
207.	HVSSR12-23	12	10.85	GAGTACTTCAGATCCGGACA	TATTACAACGGGACGCTAAT	Singh, 2009
208.	HVSSR12-25	12	12.12	AAGTTTGCAATGGAGGAATA	AAATCTTAGGCCAGGGTTAC	Singh, 2009
209.	RM27988	12	12.84	TCCAACATCATGACATCCACATCC	CATCGAAACCACCGAGTGACC	Sasaki et al.,, 2005
210.	RM28032	12	13.65	ACGACACGGATGAGTTCAGTGG	GGATCTGAGAGGAAGAGGGAAGG	Sasaki et al.,, 2005
211.	RM260	12	15.05	ACTCCACTATGACCCAGAG	GAACAATCCCTTCTACGATCG	Chen et al., 1997
212.	RM519	12	19.9	AGAGAGCCCCTAAATTTCCG	AGGTACGCTCACCTGTGGAC	Temnykh et al., 2001
213.	HVSSR12-36	12	21.17	ATCAGCGACTAAGGATCTCA	CTAATGTTGCCACATACGAA	Singh, 2009
214.	RM1226	12	27.31	TCCCTCACCCTCACTCTCAC	TCTTGTTGCTTGTGCTGTCC	McCouch et al., 2002
215.	RM2197	12	27.35	ACTGAGAACTTTAATCATCG	GAACAACTTTGAAGAGAAAC	McCouch et al., 2002
216.	RM1227	12	27.38	CATGGTAGCACACACCCTTG	CATCGACATGTGGACCACTC	McCouch et al., 2002