INVESTIGATING SALINITY TOLERANCE IN RICE THROUGH MAPPING QTLS FOR REPRODUCTIVE-STAGE TOLERANCE AND GUIDE RNA VALIDATION FOR GENE EDITING OF THE HKT FAMILY

A Dissertation

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ABSTRACT

Salinity is the most extensive problem in coastal rice eco-systems and tolerance at reproductive stage of rice is crucial for higher grain yield. In this study, two salt-tolerant varieties, CSR28 and Hasawi, and a salt-sensitive variety, BRRI dhan28, were used as parents to develop two BC_1F_2mapping populations to identify QTLs for reproductive-stage salinity tolerance in rice. Salinity of EC 10 dS/m was applied at booting stage which was constantly maintained for 20 days after leaf pruning. Data on yield, yield components and important agronomic parameters were collected from reproductive stage screening. In addition, visual salinity scoring was done using Standard Evaluation System (SES) and Na^+/K^+ was analyzed to identify the tolerant and sensitive plants. Positive and significant correlations were observed between the grain yield and number of filled spikelets in both crosses and it is negatively correlated with SES, which implies that the SES score is an initial stress indicator to identify tolerant and sensitive genotypes. Grain yield of tolerant progenies from Hasawi x BRRI dhan28 was significantly higher than that from CSR28 x BRRI dhan28. A total of 15 and 35 QTLs under salinity stress were identified through inclusive composite interval mapping (ICIM) of the crosses, CSR28 x BRRI dhan28 and Hasawi x BRRI dhan28, respectively. The QTL analysis suggested that a genomic region on chromosome 10 affects salinity tolerance at reproductive stage by increasing in number of filled spikelets, percent filled spikelet and grain yield of CSR28 x BRRI dhan28 progenies. On the other hand, for Hasawi x BRRI dhan28, chromosome 3 affects salinity tolerance by increasing productive tillers, number of filled spikelets and grain yield. These loci are good targets for marker-assisted selection aimed at improving salinity tolerance. This study also focused on targeting seven gene members of the HKT gene family, which plays a central role in determining

salinity tolerance mechanisms, via multiplex CRISPR/Cas9 based DNA free genome editing using CRISPR/Cas9 ribonucleoprotein (RNPs) and validating the gRNA designs using *in vitro* RNP assays. The results showed that cleavage activities of all genes were successful, which prepares the way for future gene editing to functionally characterize the HKT gene family.

DEDICATION

To my beloved parents:

Mina Mitra & Manoranjan Mondal

who passed many sleepless nights to keep me on track during write-up of my dissertation
&
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NOMENCLATURE

BRRI	Bangladesh Rice Research Institute
BC	Backcross
cM	Centimorgan
EC	Electrical Conductivity
FAO	Food and Agriculture Organization
HKT	High Affinity K^+ Transporters
GBS	Genotype by Sequencing
IRRI	International Rice Research Institute
KASP	Kompetitive Allele-Specific PCR
Kb	Kilobase
MAS	Marker Assisted Selection
Mb	Megabase
NHX	Na^+/H^+ Antiporter
LOD	Logarithm of Odds
PVE	Phenotypic Variation
QTL	Quantitative Trait Loci
RNP	Ribonucleoprotein
RO	Reverse Osmosis
SNP	Single Nucleotide Polymorphism
SOS	Salt Overly Sensitive
TAMU	Texas A&M University

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1. INTRODUCTION

1.1. Background

Rice is one of the most important staple cereal crops that feeds about half of the world's population (Jenkins et al., 2008) Although global rice production more than tripled between 1961 and 2010 (GRiSP, 2013); its demand is continuously increasing with the increase of global population. The crop can be grown under a wide range of agro-climatic conditions ranging from favorable to various biotic and abiotic stresses, and from irrigated to upland conditions. It has been estimated that global annual rice production needs to be increased to 850 million tons by 2025 to feed the growing population of the rice world (Khush, 1997). In addition, rice production needs to be further increased for 821 million people who are hungry today together with the additional 2 billion people expected to be undernourished by 2050 (FAO, 2020). But unfavorable environmental conditions pose a huge threat for agriculture and challenge the future food security (Kumari et al., 2009). Various abiotic stresses greatly affect the rice yield and among the abiotic stresses, salinity is the second most prevalent problem and is considered as a serious threat to increased rice production worldwide (Arzani & Ashraf, 2016; Calanca, 2017). The total area under the saline soil was 397 million ha which is 3.1% of the total land area of the world and in Asia it is about 6.3% (FAO, 2020). The salt-affected areas are expected to increase due to the adverse effect of climate change and sea-level rise (IPCC, 2019). Therefore, improving the productivity of crops in salt-stressed areas is considered essential to meet the increasing food demand.

Soil salinity is the main limiting factor in the production of rice and it is increasing due to improper irrigation practices, secondary salinization, global warming, climate change and cyclonic storms particularly in coastal areas of the world. It is also a major problem for both irrigated and rainfed agriculture whereas irrigated agricultural systems supply roughly one-third of the world's food supply (Munns, 2002). It is reported that millions of hectares in the humid regions of South and Southeast Asia are technically suited for rice production but are left uncultivated or are grown with very low yields because of salinity and problem soils (Liu et al., 2019). Therefore, there is a great urgency in addressing the problem of salinity, especially with an increasing global population and adverse effects climate change due to sea-level rise (IPCC Working Group 1 et al., 2013; IPCC 2019).

Salinity affects plant growth during all developmental stages. Presence of excessive salt in soils has harmful effects on plant growth and productivity that can significantly reduce the food production. Rice plants are highly sensitive to high concentrations of sodium and its uptake causes Na^+ toxicity and osmotic stress (Horie et al., 2009). The adverse effect of salinity stress reduces water uptake by roots and causes internal dehydration and direct accumulation of salts leads to ion toxicity that disturbs metabolic processes, particularly in photosynthetic cells (Ismail & Horie, 2017). Salt injury could be surpassed by extruding Na^+ from the cytoplasm which depends on the mechanisms of Na^+ extrusion from roots, unloading Na+ from the xylem, and by sequestration of Na^+ into vacuoles (Ismail & Horie, 2017). Salinity tolerance mechanisms are not only confined with the Na^+ exclusion or sequestration, rather the presence of cytosolic K^+ also plays an important role in salinity tolerance in rice. Besides presence of osmolytes, compatible solutes in the cytoplasm have a great effect on salinity tolerance mechanisms. (Yeo & Flowers, 1982) also reported that

leaf to leaf compartmentalization is one of the main salinity tolerance mechanisms which occur in rice. So, the knowledge of genes and metabolic or physiological networks associated with salinity tolerance will help in boosting up the breeding activities of tolerant varieties.

Rice is relatively tolerant to stress during germination, active tillering, and at maturity but is very sensitive at the early seedling stage and reproductive stage (Singh et al., 2008; Singh et al., 2010). But salinity tolerance at the seedling and reproductive stages is weakly associated suggesting that tolerance at these two stages is regulated by different sets of genes (Mishra et al., 1990; Moradi et al., 2003). The reproductive stage is crucial as it ultimately translates into grain yield. However, the importance of the seedling stage cannot be ignored as it affects crop establishment. Hence, pyramiding of contributing traits at both stages is needed for developing resilient salt-tolerant cultivars (Moradi et al., 2003, Mondal, 2014). Although salinity at the reproductive stage depresses grain yield much more than the vegetative stage but there are few studies in rice for salinity tolerance at the reproductive stage due to the difficulty of achieving reliable stage-specific phenotyping techniques (Ahmadizadeh et al., 2016). The QTL studies were focused on imposing of salt in accurate stages of interest.

Conventional breeding was used to develop stress tolerance high yielding varieties of rice. But plant selection for salt tolerance in this method was not easy because of the large environmental effects and low heritability of salt tolerance (Gregorio and Senadhira, 1993; Gregorio et al., 2002). Recently, the plant breeding methods have considerably advanced with the introduction of molecular techniques. Mainly, DNA based molecular markers are used extensively to assess the genetic diversity in most crop species. The dissection of the genetic basis of tolerance to abiotic stresses, especially in salinity, has greatly improved with

the introduction of molecular platforms that enable the identification of quantitative trait loci (QTL) governing relevant genetic variation in crops (Tanksley, 1993; Ribaut & Hoisington, 1998; Flowers et al., 2000; Koyama et al., 2001; Munns, 2005; Tuberosa & Salvi, 2005; Thomson et al., 2010; Hossain et al., 2015; Ismail & Horie, 2017; Rahman et al., 2017). Molecular markers could now be used to tag QTLs and evaluate their contributions to the phenotype by selecting for favorable alleles at these loci in a MAS scheme that aims to accelerate genetic advancement in rice (Collard & Mackill, 2008).

Single nucleotide polymorphisms (SNPs) are the markers of choice for most high throughput genotyping applications because they are ubiquitous in eukaryotic genomes, cost-effective to assay using automated platforms, and biallelic in nature, which is useful for allele calling, data analysis and data-basing (Thomson et al., 2017). SNP data could be achieved using numerous uniplex or multiplex genotyping platforms. The development of cost-effective SNP detection platforms, including KASP, TaqMan, and Fluidigm that target individual SNPs, and the low-density SNP arrays, have made use of the wealth of information published from the higher-density arrays to extract informative SNPs and invariant SNP flanking sequences that convert well to other assays (Chen et al., 2014; McCouch et al., 2010; Tung et al., 2010). In this study KASP genotyping platform will be used to detect SNPs for identifying QTL.

Identifying QTLs from a mapping population is solely dependent on the use of phenotypic and genotypic data. So aside from extracting genotypic information; establishing an accurate and precise phenotyping method is the most important step in detecting the true QTLs or genomic regions appropriate for marker-assisted breeding programs (Wang et al., 2011). This study was focused on stage-specific phenotyping, particularly salt application at

booting stage which appeared after panicle initiation. As rice varieties differ in their duration of vegetative stage, so it is very challenging to apply stress at the onset of reproductive stage. Mapping of QTLs for salinity tolerance at seedling and reproductive stages of rice can aid in the identification of genetic control of salinity tolerance leading to development of varieties with improved tolerance by precisely transferring QTLs into adapted varieties (Thomson et al., 2010).

The effect of salinity at early seedling stage in rice can be decreased by transplanting old seedlings. Although it is generally not possible to avoid stress at the reproductive stage, it is sometimes possible to use early maturing genotypes to avoid terminal salinity that only occur under coastal saline conditions (Singh & Flowers, 2020). Therefore, studies on genetic components of salinity tolerance at reproductive stage have been considered to be useful in developing high yielding rice varieties with salt tolerance. Considerable efforts have been diverted towards development of salinity tolerance at seedling stage of rice; however, few attempts have been made to identify QTLs associated with the reproductive stage salinity tolerance (Hossain et al., 2015; Zeng et al., 2002). Therefore, it is necessary to develop salinity tolerance at the reproductive stage of rice; without which total crop failure may occur with huge investment loss in rice production and be a threat to global food security.

The recent advances in biotechnology and molecular breeding have brought tremendous changes in rice productivity across the globe with the development of improved varieties (Mishra et al., 2018). Genome editing with CRISPR/Cas 9 plays a vital role in improvement and understanding the gene functions associated with abiotic stresses like salinity in rice and provides opportunity for plant improvement. Further, the CRISPR/Cas 9 system has become more powerful with the introduction of multiplex genome editing which

can target multiple genes with single gRNA or express multiple gRNAs from a single transcript and also efficient DNA free genome editing with CRISPR/Cas9 ribonucleoprotein (RNP) because of having fewer off-target mutations. HKT is a promising salt-tolerant gene, and plays a vital role in the tolerance to salinity stress (Almeida et al., 2013). There are seven gene members reported for the HKT family; the members of the *HKT* gene family play a central role in controlling Na^+ accumulation and also determine the mechanisms of salinity tolerance. To achieve CRISPR-mediated gene editing for HKT gene family, a functional Cas9–gRNA ribonucleoprotein (RNP) complex must be present inside the nucleus and direct delivery of this RNP complex could be the most straightforward option. This is a widely acceptable method for producing genome-edited plants in a short period of time and has a good prospect of being commercialized.

1.2. General Objective

The general objective was to identify quantitative trait loci (QTLs) for salinity tolerance at the reproductive stage of rice plant using BC_1F_2 populations derived from two salt-tolerant varieties; CSR28 (Indian variety) and Hasawi (Saudi Arabian variety) and a salt-sensitive Bangladeshi variety, BRRI dhan28; and preparation for genome editing of the HKT gene family thorough designing an approach for multiplex CRISPR/Cas9 based gene editing targeting seven gene members of the HKT family of sodium transporters.

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2. REVIEW OF LITERATURE

2.1. Influence of Soil Salinity on Growth and Development of Plants

Salinity is one of the most detrimental environmental factors that limit the crop productivity as most of the plants are sensitive to salinity caused by high concentrations of salts in the root zone of the crops (Shrivastava & Kumar, 2015). Salinization is the accumulation of water-soluble salts in the soil profile to a level that impacts crop production to various degrees. A soil is considered to be saline if the electrical conductivity of its saturation extract (ECe) is above 4 dS/m at 25 ^{0}C, an exchangeable sodium percentage (ESP) is less than 15, and a pH is less than 8.5 (US Salinity Laboratory Staff, 1954). Katsuhara et al., (2008) and Chaumont & Tyerman, (2014) reported that plant roots have lower water potential than the outside environment leading to an influx of water through channels known as aquaporin during normal or control conditions; while during salinity stress, soil water potential is reduced incapacitating the root's ability to uptake water and causes water deficit (Pardo, 2010; Roy et al., 2014).

Soil salinization can be caused by both natural and artificial processes such as mineral weathering, fertilization, irrigation and surface runoff (ASCE, 1990; Somani, 1991). Scarcity of water and hot dry climates frequently cause salinity intrusions that limit or prevent crop production. Soil salinity increases due to capillary transport of water from a salt-laden water table or saline groundwater and then accumulates on the soil surface due to evaporation. In the coastal zone, the soil becomes saline due to direct inundation by seawater, upward or lateral movement of saline groundwater and evapotranspiration from the crop field (Bhumbla and Abrol, 1978; Somani, 1991). Globally, the total area of saline

soils is 397 million ha and sodic soils is 434 million ha at the global level (FAO, 2005), which is more than 6% of the world's total land area. FAO (2008) estimated that about 20% (45 million ha) of irrigated land and 2% (32 million ha) of dryland agriculture are salt-affected lands. These problems pose the greatest threat to increase food production to meet the increasing demand of the growing population in Asia (Abrol, 1986).

The presence of salt in the soil solution adversely affects the growth and development of plants owing to reduced water uptake and nutrient imbalances (Munns et al., 2006). Among the soluble salts, NaCl is the most abundant salt that causes soil salinity (Türkan & Demiral, 2009). The effect of soil salinity can be classified as osmotic, toxic or nutritional. Salt toxicity is considered as the primary salt injury, whereas osmotic and nutritional stresses (including deficiency of other nutrients) are considered secondary salt-induced injuries (Manneh, 2004). Salinity inhibits plant growth by osmotic effect, which reduces the ability of the plant to uptake water (Munns, 2002). It also inhibits uptake of phosphorus, potassium, nitrate, and calcium that leads to ion toxicity and oxidative stress (Tuteja et al., 2012). On the other hand, a high concentration of salts in the soil profile makes it difficult for the roots to extract water (Munns & Tester, 2008). The most important effects of salinity on the plants are lowering down the water potential, ion toxicity, specifically Na^+ and Cl^-, and interference with the uptake of essential nutrients. Salt stress in the soil is often associated with other abiotic stresses. Therefore, it is important to consider multiple stress tolerance during the breeding process for the development of salt tolerant rice varieties (Gregorio et al., 2002).

2.2. Ionic Stress

Restriction of Na^+ entry into the root cell and the transpirational stream is a fundamental issue to prevent the accumulation of toxic levels of salt in the shoot (Tuteja et al., 2012). Munns et al., (1999) mentioned that about 97% of the Na^+ should be excluded by plants from the root surface and as a result preventing toxic levels of Na^+ accumulation in the shoots. Basically, increased Na^+ accumulation into the plant due to high concentrations of Na^+ in the soil causes ionic stress in plants. (Bartels & Sunkar, 2005) suggested three mechanisms that prevented excess Na^+ accumulation into the roots. They are; 1) restriction of Na^+ entry and infiltration into the plants by Na^+ transporters. 2) compartmentalization of the Na^+ into the vacuoles and 3) extrusion of Na^+: cytosolic Na^+ can be transported back to the external medium or the apoplast via plasma membrane Na^+/ H^+ antiporter activity.

2.2.1. Ion Selectivity

In saline conditions, the degree of selectivity is the major concern of any solute transport by plants, especially between potassium and sodium (Ashraf et al., 2005). One of the most important physiological mechanisms of salt tolerance is the selective absorption of K^+ by plants from the saline soil (Ashraf et al., 2006). Na^+ is the major component of salt stress and controlling the entry of Na^+ into the roots could avoid ionic stress. The difference between salt-tolerant and salt-sensitive plant species is controlling the controlling of Na^+ and K^+ transport and uptake to the leaves. Maintaining better concentrations of K^+ and Ca^{++} and restricting the uptake of the Na^+ is the main salt-tolerant mechanisms of plants.

2.2.2. Na^+ Exclusion

Na^+ exclusion is the most important strategy of salt tolerance mechanisms. Transport of Na^+ into the leaves and then excreted out of the plant tissue to keep the Na^+ concentration lower particularly in younger leaves in the major mechanisms of salt- tolerant plant species (Tuteja et al., 2012). As Na^+ is translocated through the transpirational stream, the older leaves will have much higher Na^+ than the younger leaves because its concentration increases with time.

2.2.3. Na^+ Sequestration

Na^+ sequestration in vacuoles is another mechanism to control the presence of Na^+ into the cytoplasm and due to this activity, plants survive under salinity stress. The central vacuole plays a vital role in the regulation of cytoplasmic ion homeostasis (Tujeta et al., 2012). The excess salt present in the cytoplasm tends to cause the plant to be more salt-sensitive. That is why Na^+/H^+ antiporter activities at the tonoplast and proton motive gradient force help to balance the Na^+ concentration into the cytoplasm by vacuolar H^+ translocating enzymes, H^+ ATPase and H^+ inorganic pyrophosphate (PPiase) and combination of downhill movement of H^+ with the uphill movement of Na^+ against the electrochemical potential (Blumwald & Gelli, 1997).

2.3. Osmotic Stress

External salinity decreases water flow into the plant, restricts the water uptake to cells, and reduces the turgor potential and cell volume (Tal, 1984). In general, plants maintain a higher K^+/Na^+ ratio in normal conditions, but in stress conditions, the ratio

decreased (Tuteja et al., 2012). Tuteja et al., (2012) also mentioned that K^+ provides the required osmotic potential for water uptake by plant cells. So, it is an important element for the maintenance of biochemical processes under salinity stress. Salt stress that affects water supply leads to changes in stomatal opening that can set in motion a chain of events originating from a decline in the leaf internal CO_2 concentration, consecutively inhibiting the carbon reduction cycle, light reactions, energy charge, and proton pumping (Kaiser, 1979).

2.3.1. Osmotic Adjustment

Under osmotic stress, plants need to maintain internal water potential lower than soil water potential because of the water potential always tends to move from higher to lower. To maintain this potential flow, osmotica need to be increased, either by uptake of soil solutes or by the synthesis of compatible solutes (Parvaiz & Satyawati, 2008). These organic or compatible solutes protect plants from salt stress by (i) osmotic adjustment, which helps in turgor maintenance; (ii) detoxification of reactive oxygen species; and (iii) stabilization of the quaternary structure of proteins (Bohnert & Jensen, 1996). Generally, compatible solutes that are composed of simple and complex sugar, sugar alcohols, proline, glycine betaine etc. are highly polar and solubilized into the water of the plant cell where they could relate with the macromolecules. According to *in vitro* experiments, different compatible solutes have different functions, yet the main function is to stabilize protein or protein complex under stress conditions.

2.3.1.1. Osmotic Adjustment by Proline

Most of the plants synthesize and accumulate proline during salinity stress. Generally, accumulation of proline occurs in cytosol so that it contributes to cytoplasmic osmotic adjustment (Ketchum et al., 1991); because it is important to keep a higher K^+/Na^+ ratio in the cytoplasm. Proline is an amino acid and it is synthesized by glutamic acid by the action of pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). Kishor et al., (1995) reported that overexpression of the P5CS gene in transgenic tobacco increases the production of proline and as a result plants become salinity/drought tolerance. Also salt stress increases proline utilization in the apical region of the barley root. Exogenous application of prolines provides osmoprotectant and helps the growth and development of salinity stressed plants (Tujeta et al., 2012).

2.3.1.2. Osmotic Adjustment by Glycine Betaine (GB)

The organic compound glycine betaine is mainly located in chloroplasts and plays an important role in chloroplast adjustment and protection of thylakoid membranes. As a result, photosynthetic efficiency and plasma membrane integrity is maintained (Yokoi et al., 2002). Glycine betaine is synthesized by plants via two-step oxidation of choline. They are choline to betaine aldehyde, then betaine aldehyde to glycine betaine. The first reaction is catalyzed by ferredoxin-dependent choline monooxygenase (CMO) and the second one is catalyzed by an NAD^+ dependent betaine aldehyde dehydrogenase (BADH) (Chen & Murata, 2002). GB helps in response to stress in many crops, like spinach, barley, tomato, potato, rice, carrot, and sorghum (Yang & Poovaiah, 2003). Under the saline condition, GB protects the photosystem II complex by stabilizing the association of the extrinsic PSII

complex proteins (Murata et al., 1992). Even foliar application of GB significantly improves the salt tolerance in rice plants (Lutts, 2000) and fruit yield tomato, which is exposed to either salt stress or high temperatures increased about 40% compared to untreated plants with GB (Mäkelä et al., 1998). For tolerant plants, GB improves the osmotic stress of transgenic plants (Hayashi et al., 1997).

2.4 Root Water Relationship during Stress Condition

Osmotic and hydrostatic forces regulate the root water uptake rate. Root water transport is divided into radial and axial transport (Kramer & Boyer, 1995). After the water has been absorbed by root hairs, it moves through the ground tissue by three pathways. They are: (1) symplast i.e. water moves from the cytoplasm of one cell to another via plasmodesmata, (2) transmembrane pathway i.e. water moves through the cytoplasm and the vacuoles crossing the plasma membrane and vacuolar membranes, and lastly (3) apoplast where water moves through the pores between the fibrils of the cell wall and through the intercellular spaces (Kramer & Boyer, 1995). Water that moves via the apoplast does not encounter any barrier until it reaches the endodermis. But endodermis has a waxy region (casperian strips), which force water to cross the plasma membranes of endodermal cells instead of slipping between the cells. Apoplastic barriers for water and ion flow will be developed during the hyperosmotic condition (Stasovski & Peterson, 1991). Generally, root water uptake and root hydraulic conductivity (L) decreases upon exposure to hyperosmotic potential and by osmotic shock as a result of an aquaporin conformational change caused by negative pressures (Wan et al., 2004).

The decrease of L could be a strategy to reduce water flow from roots to soil as soil osmotic potential is lower than the roots (Stasovski and Peterson, 1991). During hyperosmotic stress, the formation of suberin lamellae in the endo and exodermis could result in localized high resistances for water and ion flow in the root apoplast. The exodermis could contribute to the regulation of water uptake into roots (Stasovski and Peterson, 1991).

Water potential is defined as the summation of solute potential and pressure potential which can be expressed as $\Psi w = \Psi s$ (solute/osmotic potential) + Ψp (pressure potential) (Kramer & Boyer, 1995). In order for water to move through the plant from the soil to the air (transpiration), Ψ^{soil} must be $>\Psi^{root} >\Psi^{stem} >\Psi^{leaf} >\Psi^{atmosphere}$. The internal Ψ**w** of a plant cell is more negative than pure water potential because of the cytoplasm's high solute content. So, water will move from the soil into a root cells via osmosis and the flow order is Ψ^{soil} must be $>\Psi^{root} >\Psi^{cortex} >\Psi^{stele}$. But, in hyperosmotic condition, $\Psi^{root} >\Psi^{soil}$, so dehydration occurs within the root cell (Kramer & Boyer, 1995).

Growth sustaining Ψw depends on two things: (1) Ψw must be sufficiently low to move water to a growing tissue/cell from the source, and (2) must cause the water to enter into the cell (Boyer & Silk, 2004). Small water potential differences are required to cause a growth-sustaining water flux to enter into a cell.

In xylem and phloem, solute flow is driven by negative and positive hydrostatic pressure (Kramer & Boyer, 1995). But outside of the xylem and phloem, gradients in potential should exist. The main aspect of producing a growth-sustaining water potential pattern is a radial gradient in solute potential. Another aspect is to consider pressure-driven-flow from phloem because some of the water comes from phloem for root growth (Boyer &

Silk, 2004). Under stress conditions, turgor recovery is faster in the deeper root cortex rather than surface area as radial water movement occurs from both the surrounding soil (inward flux) and the functional phloem (radial and longitudinal flux) in response to both water potential gradients and a pressure-driven conductive flow.

Root growth zone and their rhizosphere have some interaction in terms of water uptake. Water extraction patterns in the rhizosphere of the growth zone indicates that water flows radially from the soil into the growth zone of roots (proximal part) and flux decreases in magnitude as the water moves inward (Boyer & Silk, 2004). In the homogeneous soil, growth-rate patterns of the root are often quasi-steady. While a steady field of water content is found around the moving growth zone, which implies growing root tips are surrounded with a micro-environment of soil moisture as they penetrate the soil (Kramer & Boyer, 1995). Since water for transpiration is absorbed well behind the growth zone, the growth zone is protected from transpiration-induced dryness.

2.5. Mechanisms of Salt Tolerant in Rice

Crop performance is affected by different abiotic stresses; among them, salinity is the second most prevalent soil problem in the rice-growing countries (Greogrio et al., 1997). Tolerance to salinity is genetically and physiologically complicated and inherited quantitatively. Yeo &Flowers (1984) mentioned that the mechanisms lead to low Na^+ content in the functional tissue, leading to low Na^+-K^+ ratio in the shoot. Gregorio & Senadhira. (1993) observed the direct relation of a low Na^+-K^+ ratio to salinity tolerance. There are several mechanisms of salinity tolerance, but tolerant varieties often have one or

two of the mechanisms (Yeo & Flowers, 1984). Therefore, physiological characteristics need to be improved for increasing salinity tolerance of plants.

Although breeding and biotechnological activities have a major role in the crop improvement from salinity stress; the plant has their own defense mechanisms to protect themselves from the salt stress. Several Na^+ transporters are involved in the defense mechanisms that detoxify the high level of Na^+ concentration. The major transporters/genes related to salt tolerance are high-affinity K^+ transporters (HKT), Na^+/H^+ exchanger (NHX) and salt overly sensitive (SOS).

2.5.1. Mechanisms Mediated by High-Affinity K Ion Transporters (HKT)

HKT plays a vital role in salt-tolerance and root to shoot Na^+ partitioning (Deinlein et al., 2014). After sequence and transport analysis, there are two subgroups that belong to the HKT gene family; they are class 1, which is xylem parenchyma localized and class 2 transporters. Class 1 transporters consist of OsHKT1;1, OsHKT1;3 OsHKT1;4 OsHKT1;5 which are Na^+ selective transporters, and class 2 transporters includes OsHKT2;1 OsHKT2;3 OsHKT2;4 which are Na^+-K^+ co transporters. To reduce the transfer and accumulation of Na^+ in shoots during salt stress, the control of net Na^+ loading into the xylem is also important. Besides, Ismail & Horie, (2017) mentioned that Na^+ unloading from the xylem is the best mechanism to achieve low Na^+ concentrations in the xylem sap under salt stress. Different genes are responsible for these mechanisms and their expression area as well as the stage of expression is also different (Table 2.1).

Table 2.1. Genes and mechanisms of expression in Na^+/K^+.

Gene name	Expression area	Stage of expression	Functions
OsHKT1;1	Vicinity of xylem and phloem	Vegetative and reproductive	1. Na^+ unloads from the xylem vessel to xylem parenchyma. 2. Na^+ loading into the phloem of leaves for Na^+ recirculation, and phloem-mediated Na^+ recirculation from shoots to roots or from younger leaves to older leaves is another mechanism to regulate the presence of Na^+ in the younger leaves (Ismail & Horie, 2017).
OsHKT1;3	Leaf blade, root and vascular tissue	Vegetative	1. It mediates both inward and outward Na^+ currents with weak inward rectification.
OsHKT1;4	Stem and leaf sheath	Reproductive	1. Na^+ sequestrates in the leaf sheath and stem to protect Na^+ transfer into the leaf blade (Hamamoto et al., 2015; Ismail & Horie, 2017)
OsHKT1;5	Shoots	Reproductive	1. Na^+ excludes from the xylem sap to xylem parenchyma, so leaves are protected from Na^+ toxicity. 2. Na^+ loaded into the phloem cell and due to its downstream nature, Na^+ transported back to the root. As a result, it prevents Na^+ over-accumulation in shoots. 3. K^+ is released to xylem vessel from xylem parenchyma due to membrane depolarization by KOR and NOR channel (Horie et al., 2009).
OsHKT2;1	Roots	Vegetative and reproductive	1. Na^+ influx is regulated due to K^+ starvation condition and as a nutrient of the substitution of K^+(Horie et al., 2009).
OsHKT2;3	Leaf blade, sheath, root	Vegetative and reproductive	1. It transports Na^+-K^+ together i.e. cotransporter. OsHKT2;3 and OsHKT2;4 are very similar in function and 93% amino acid sequence is identical to OsHKT2;4 (Horie, Brodsky, et al., 2011).
OsHKT2;4	Plasma membrane of rice root hair cell	Vegetative and reproductive	1. Cation transport activity including Ca2+ (Horie et al., 2012). 2. Strong K+ selectivity over divalent cation and low Na+ transport activity (Horie, Sugawara, et al., 2011)

2.5.2. Mechanisms Mediated by Na^+/H^+ Exchanger (NHX)

In plants, for energizing the secondary active transport of ions and metabolites, H^+ electrochemical gradients are generated by the H^+-ATPase at the plasma membrane or the V- ATPase and PPase in the intracellular compartments (Bassil & Blumwald, 2014). Any cation/H^+ exchangers use the H^+ gradient to combine the passive transport of H^+ to the movement of cations against their gradient. The coupled exchange of Na^+ for H^+ is driven by Na^+/H^+ antiporters (NHXs), particularly in plants. The mechanisms of the tonoplast localized NHXs in the plant system is the sequestration of Na^+ into vacuole or endosome in exchange of H^+ efflux to cytosol to keep the cytoplasm less toxic and also Na^+ efflux out of the cell in swapping for H^+ influx into the plasma membrane (Bassil & Blumwald, 2014). NHX transporters are localized into the vacuole and also in the endosome. These transporters are responsible for osmoregulation, cell growth and plant development whereas endosomal ones are for vesicular trafficking, protein processing and cargo delivery (Deinlein et al., 2014). They are essential for Na^+ detoxification via Na^+ sequestration within the vacuole. According to a recent study, NHX type proteins compartmentalize K^+ into the vacuole and aids in cellular pH homeostasis. Rice contains seven isoforms belonging to three classes; two divergent members located at the endosome, four in the intracellular isoforms i.e., located in the vacuole and one member is in the plasma membrane (Table 2.2). In plants, NHX genes involve both Na^+/H^+ and K^+/H^+ exchange that affect both salinity tolerance and K^+ nutrition. According to Villalta et al. (2008) salt sensitivity increased due to the reduction of V-ATPase activity in the trans-Golgi network/ early endosome (TGN/EE). But interestingly, Undurraga et al. (2012) said that overexpression of vacuolar-type 1 H^+-PPase AVP1 improves plant salt tolerance by intervening in the vacuolar Na^+ sequestration.

Table 2.2. Transporters involved in Na^+/H^+ exchanger mechanisms.

Gene name	Localization	Functions
NHX1, NHX2, NHX3 and NHX4	Vacuole	1. Na+/H+ exchange activity tends Na+ sequestrate into vacuole (A. M. Ismail & Horie, 2017) from cytosol to keep the cytosol less toxic. 2. Accumulate high K+ concentration in vacuole (Ismail & Horie, 2017).
NHX 5 and NHX 6	Golgi apparatus and trans-golgi network and pre-vacuolar compartment	1. Endomembrane pH homeostasis is mediated by these transporters which are important for association of vacuolar trafficking receptors and cargo proteins (A. M. Ismail & Horie, 2017) because trans-golgi network is more acidic (Bassil & Blumwald, 2014). 2. Maintain the process of protein trafficking from the golgi apparatus and trans-golgi network to vacuoles (Ismail & Horie, 2017).
NHX 7	Plasma membrane	1. Reduce cytoplasmic Na+ by Na^+ efflux out of the cell in exchange for H^+ influx into the cell (Bassil & Blumwald, 2014).

2.5.3. Mechanisms Mediated by Salt Overly Sensitive (SOS)

The prominent increase in cytoplasmic Na^+ disturbs enzymatic functions and is noxious to both cells and the whole plant system. Under the non-saline condition, Na^+ uptake is mediated by HKT ion transporters, but when the amount of Na^+ increases into the surrounding environment, it enters into the plant through the plasma membrane via non-selective cation channels (NSCC) whose molecular identity is still unknown or via anatomical leaks in the root endodermis. The entrance of Na^+ can be controlled by minimizing Na^+ entry into the cell, maximizing the compartmentalization of Na^+ into the vacuole and increasing the efflux of Na^+ from the cell. One of the main responses to salt stress is maintaining cellular ion homeostasis or maintaining the proper equilibrium of K^+ or Ca^{2+} by limiting the accumulation of toxic sodium (Na^+) (Clarkson & Hanson, 1980; Tester

& Davenport, 2003). SOS signaling pathway is well-defined for maintaining ion homeostasis. Isolation and characterization of several mutants that showed root growth hypersensitivity under salt stress led to the identification SOS signaling pathway (Zhu et al., 1998). Activation of this pathway helps to exclude Na^+ from the cytosol. This pathway includes SOS1, SOS2 and SOS3 (Table 2.3). The mechanisms of SOS signaling pathway are given below:

1. Extrusion of Na^+ from the cytoplasm
2. Enhance shoot salt tolerance and ion homeostatic regulation in protecting different tissues and organs against salt stress (Ji et al., 2013).
3. Structural changes in root-like emergence of lateral root, modification of root hair and a significant role in the plastic development of root hairs under salt (Wang & Li, 2008; Wang et al., 2008).

Table 2.3. Signaling pathways in SOS mechanisms.

Gene name	**Expression**	**Functions**
SOS 1	Root and shoot (Liu et al., 2000; Quan et al., 2007; Shi et al., 2002)	Na+ extrusion from the cytosol with H+ ATPase activity and Na+/H+ antiporter activity (Ismail & Horie, 2017).
SOS2	Root and shoot (Liu et al., 2000; Shi et al., 2002; Quan et al., 2007)	Lateral root development during salt stress by inducing auxin biosynthesis (Ji et al., 2013).
SOS 3	Root (Liu et al., 2000; Shi et al., 2002; Quan et al., 2007)	Trigger Ca2+ sensor with the increase of Na+, as a result cytosolic Ca^{2+} increased, which is important for tolerance (Ji et al., 2013). Lateral root development during salt stress-inducing auxin biosynthesis.

A combination of physiological mechanisms for overall salinity tolerance is logically a desirable long-term objective (Yeo & Flowers, 1982). It is essential to maintain ion homeostasis in the cytoplasm to have salt tolerance in plants. Gorham, (1993) reported an association between salt tolerance and Na^+ exclusion. Salt tolerance relies upon the ability of the plant to establish and maintain a new cell membrane electrical potential at a tolerable cytoplasmic ion homeostasis. The exclusion of Na^+ in the roots ensures that Na^+ will not accumulate beyond toxic levels within the leaves. The premature death of older leaves is observed corresponding with a failure in Na^+ exclusion which manifests its toxic effect after days or weeks of accumulation of Na^+ (Thapa, 2004).

2.6. Channels that Mediate Invasive Na^+ Influx into Roots during Salt Stress

Na^+ efflux or exclusion from the shoots or Na^+ influx into roots in a unidirectional way has a large impact on Na^+ accumulation in root under high saline conditions. Mainly, toxic Na^+ influx is passively mediated by voltage-independent (or weakly voltage-dependent) nonselective cation channels (NSCCs) in plants because of the electrophysiological properties of Na^+ currents (Ismail & Horie, 2017). Based on their voltage dependency, NSCC can be divided into three subgroups. They are voltage-insensitive NSCCs (VI-NSCCs), depolarization-activated NSCCs (DA-NSCCs), and hyperpolarization-activated NSCCs (HA-NSCCs). Among these three channels VI-NSCCs mediates a major portion of toxic Na^+ influx and also divalent cations like Ca^{2+} influx into the roots (Ismail & Horie, 2017). VI-NSCC is also permeable to K^+, which plays a vital role in salt tolerance. Demidchik et al., (2002) found that VI-NSCC-mediated Ca^{2+} transport contributes to Ca^{2+}acquisition, which were involved in the growth of *Arabidopsis* roots.

When the level of Na^+ increases in the soil, VI-NSCCs is the vital source for the Na^+ entry into the roots. But Ca^{2+} exhibits a strong blocking effect on Na^+ influx into intact roots (Essah et al., 2003; Tyerman et al., 1997). This characteristic could somewhat account for the ameliorative effect of increasing external Ca^{2+} on plant salt tolerance (Demidchik & Maathuis, 2007; Rains & Epstein, 1967).

Two important families, i.e. cyclic nucleotide-gated channels (CNGCs) and ionotropic glutamate receptors (GLR), play an essential role in up taking up monovalent and divalent cations into the roots. Especially, GLRs can mediate Ca^{2+} currents in pollen tubes. Recently, two novel channels, i.e., REDUCED HYPEROSMOLALITY–INDUCED [Ca^{2+}] INCREASE 1 (OSCA1) and CALCIUM PERMEABLE STRESS-GATED CATION CHANNEL 1 (CSC1) were found to mediate Ca^{2+}currents in response to osmotic stress in *Arabidopsis* (Yuan et al., 2014).

2.7. Channels and Transporters that Affect K^+ Homeostasis and Salt Tolerance

K^+ has its own importance for salt tolerance mechanisms as like as Na^+, because maintaining a higher K^+/Na^+ ratio in leaves is linked with the salt tolerance of plants (Hauser & Horie, 2010). K^+ efflux systems is considered as an important mechanism because of their deep relevance for K^+ retention capacity during salt stress (Demidchik, 2014; Shabala et al., 2006). A strong correlation between the salt-stressed root and salt tolerance was found by Shabala & Cuin (2008) and implies the importance of K^+ homeostasis. Maintaining high cytosolic K^+/Na^+ ratios in leaf blades during salt stress is essential for salt tolerance. Under the saline condition, some varieties of barley can retain a higher K^+ content in leaves that exhibited better K^+ retention ability i.e., lower K^+ efflux activity (Wu et al., 2015). A similar

assessment was also observed in wheat varieties indicated that the lowering down the activity of salt-induced K^+ efflux from the leaf mesophyll cells intensely correlates with overall salt tolerance. These results imply that K^+ retention ability in leaf mesophyll cells can maintain high cytosolic K^+/Na^+ ratios resulting in increased salt tolerance of the plants. There are two members of a K^+ transporter gene families in rice, named Oryza sativa high-affinity K^+ (OsHAK1) and OsHAK5, are upregulated by salt stress and associated with salt tolerance. These genes help K^+ uptake and maintain high K^+/Na^+ ratios during salt stress (Ismail & Horie, 2017).

2.8. Effects of Salinity on Rice

Rice plants have a salinity threshold of 4 dS/m, above which yield loss occurs (Maas & Hoffman, 1977). Plant cells have the ability to compartmentalize salt ions, like Na^+ and Cl^- in vacuoles when they reach damaging levels (salt stress). When salt ions are sequestered in vacuoles, K^+ and organic solutes (most commonly glycine, betaine and proline) accumulate in the cytoplasm and organelles to balance the osmotic pressure of the ions in the vacuole (Lutts et al., 1999). The amount of accumulation varies from one plant species to another. Rice, a salt-sensitive crop species, is relatively ineffective in controlling the influx of Na^+ and Cl^- ions to the shoot (Yeo and Flowers, 1982), and cannot accumulate glycine betaine (Rathinasabapathi et al., 1993).

Although rice shows its highest tolerance to salinity at germination and maturation, salt stress in all developmental stages of rice can contribute to yield losses (Moradi et al., 2003). The effect of salinity on rice depends on the kind and level of salinity, duration of exposure, cultivar, crop growth stage, water regime, soil physical properties, temperature,

and solar radiation (Neue et al., 1998). Salinity stress symptoms include white tips of affected leaves, chlorotic patches on some leaves, plant stunting, reduced tillering, patchy field growth, and in severe cases, plant death. Salinity significantly reduces the tiller number per plant, spikelet number per panicle, fertility, panicle length, and primary branches per panicle (Cui et al., 1995; Heenan et al., 1988; Khatun & Flowers, 1995; Zeng et al., 2002).

The number of spikelets per panicle is the most important yield component for salt tolerance. This component is determined at the early reproductive stage, around panicle initiation (PI) (Counce et al., 2000). The loss in spikelet number per plant was most significant when the stress was imposed before PI (3-leaf stage) or between PI and booting stage (~16 days after PI). This loss of potential spikelets is attributed to the degeneration of primary and secondary branches and flower primordial (Zeng et al., 2001).

Yeo & Flowers (1986) found that during reproductive development, tolerant genotypes tended to exclude more salt, hence less salt concentration in flag leaves and developing panicles, resulting in higher grain yield. Rao et al. (2008) reported grain yield reduction by 27 %, 46 % and 50 % at an ECe of 8 dS/m in tolerant, moderately-tolerant and susceptible rice cultivars, respectively. Across three tolerance cultivars, grain yield was reduced by only 9.4 %.

Salinity tolerance at the seedling and reproductive stages is only weakly associated (Moradi et al., 2003); suggesting that a different set of genes regulates tolerance at these two stages. The reproductive stage is crucial as it ultimately determines grain yield; however, the importance of the seedling stage cannot be ignored as it affects crop establishment. Hence, a pyramiding of contributing traits at both stages is needed for developing resilient salt-

tolerant cultivars (Moradi et al., 2003). Salinity at the reproductive stage depresses grain yield much more than the vegetative stage.

Considerable variation for salt tolerance at critical stages in the cultivated gene pool was also reported by other workers (Moradi et al., 2003; Yeo & Flowers, 1982). This genetic variability can be utilized for the improvement of salt tolerance by focusing on specific yield components. Nevertheless, the underlying genes conferring tolerance during PI are at present unknown (Walia et al., 2007). Studies showed that the typical mechanism of salt tolerance in rice was Na^+ exclusion or reduction of Na^+ uptake and increased absorption of K^+ to maintain a low Na-K ratio in the shoots (Gregorio et al., 1997; Lee et al., 2003).

2.9. KASP Genotyping

The KASP genotyping assay uses a unique form of competitive allele-specific PCR which is combined with a novel, homogeneous, a fluorescence-based reporting system for the identification and measurement of genetic variation that occurs at the nucleotide level to detect single nucleotide polymorphisms (SNPs) or insertions and deletions (InDels) (He et al., 2014). Cost is a major factor that determines whether or not a marker-assisted selection (MAS) is a viable breeding method for the breeders (Steele et al., 2018). Although MAS has some advantages like improved reliability, it will hardly be used if it is more expensive than phenotyping; while reducing the costs of markers could increase the use of MAS (Steele et al., 2018). Kompetitive allele-specific PCR (KASP) is a cost-effective and flexible exclusive technology of LGC Genomics, and it is more effective than chip-based Illumina Golden Gate and BeadXpress platforms in terms of precise allele calling, less genotyping error, and lower genotyping costs (Semagn et al., 2014). It has been utilized over many years to drive research

targeting the genetic improvement of animals and field crops. KASP technology is more rapid than SSRs and makes it suitable for a wide range of experimental designs with greatly varying target loci and sample numbers (He et al., 2014).

KASP has been established in several plant-breeding applications, such as quality control analysis of germplasm (Ertiro et al., 2015; Semagn et al., 2012), screening for candidate alleles and genotyping (Mideros et al., 2014; Pham et al., 2015), bulk segregant analysis and genetic (QTL) mapping (Mackay et al., 2014; Ramirez-Gonzalez et al., 2015) and MAS (Cabral et al., 2014; Leal-Bertioli et al., 2015). To analyze crosses between *Oryza sativa* ssp. *indica* and *Oryza glaberrima*, 2,015 KASP assays were made widely available for rice (Pariasca-Tanaka et al., 2015) that were developed using an array-based Illumina GoldenGate technology by the Generation Challenge Program of the Consultative Group for International Agricultural Research (CGIAR). LGC Genomics from the UK provide a full KASP genotyping services and the KASP reagents can be ordered for carrying out assays in basic molecular laboratory work.

In the first stage of PCR, one of the allele-specific primers binds to its target SNP with the common reverse primer, amplifying the target region (He et al., 2014; LGC, 2013). As PCR proceeds, one of the fluor-labeled oligos is also incorporated into the template, which is complementary to the new tail sequence of the amplified allele and is hence no longer bound to its quencher-labeled complement. The appropriate fluorescent signal is generated as the fluor is no longer quenched. If the genotype at a given SNP is homozygous, only one or the other of the possible fluorescent signals will be generated. A mixed fluorescent signal will be generated if the individual is heterozygous (LGC, 2013).

2.10. Skim Sequencing

Genotyping by sequencing (GBS) is a comparatively new method used to determine the differences in the genetic makeup of individuals (Golicz et al., 2015). Genotyping and next-generation sequencing are the two basic methods for GBS. GBS protocols can take multiple forms depending on the objective of the study, including sequencing of the DNA from the individuals of interest (two parents of the bi-parental mapping population and their progeny), mapping of the sequencing reads to the reference sequence, SNP calling and filtering, SNP genotyping and imputation, haplotype identification and downstream analysis. General marker discovery, haplotype identification, and recombination characterization to quantitative trait locus (QTL) analysis, genome-wide association studies (GWAS), and genomic selection (GS) could also be performed by GBS. This technology has already been applied to different plant species including rice, maize, artichoke, and Arabidopsis thaliana. It is a favorable approach that is likely to provide new and important insights into plant biology.

While traditional GBS uses restriction enzyme (RE) digestion for reduced representation sequencing, skim sequencing directly sequences random sites at low coverage, and can be used for biparental QTL mapping, even when progeny samples are sequenced with 1X coverage on average. Parental reads were aligned to an established reference genome, and SNPs were discovered based only on the reads (Golicz et al., 2015). The resulting SNPs list is used for genotyping of the progeny reads. Where there were no reads, for a progeny sample, at a SNP location, the genotype was called based on an imputation technique, which uses the haplotype structure of the parents.

There are advantages of skim sequencing over the traditional GBS, which was described by Scheben et al. (2018). In short, the library preparation is much easier with the decreased number of steps, reduces the problems of downstream analysis, eliminates biases caused by the restriction enzymes, the cost is also lower and the SNP discovery rate is higher.

2.11. QTL Mapping

Most of the economically important traits like yield are controlled by several genes or polygenes. These are quantitative traits and are highly dependent on the environment. The genes control a particular trait and are described to have minor and major effects. These genes or the location of the genes can be identified with the aid of molecular markers (Alberts et al., 2007).

Quantitative Trait Loci are a piece of DNA containing the loci or regions in a genome that control a quantitative trait. Recent cloning and characterization of several QTLs indicated that one QTL might contain one gene; however, it may contain more than one genes. Chahal and Ghosal (2002) suggested that defining and studying the entire locus of genes associated with a trait may give the basis of understanding their effects to a particular individual genotype concerned. Collard et al. (2005) mentioned the advantages of QTL mapping: a) provides a fundamental understanding of individual gene and its interactions; b) enables the possibility for positional cloning; c) may improve breeding value estimate and selection response through marker-assisted selection.

A mapping population is required for QTL analysis and QTL analysis includes genotyping as well as phenotyping of the trait of interest. A mapping population is generated from a cross between two different parents where each of the parents is contrasting for the

trait of interest. It can consist of segregating populations such as F_2, F_3, or backcross (BC) populations that provide of all the possible genotypes and will cover the QTL across genotypes. In conducting QTL analysis, phenotyping and genotyping data are used to obtain a QTL map. Aside from the segregating mapping populations, there are some permanent mapping populations like recombinant inbred lines (RILs), near-isogenic lines (NILs), and doubled haploids (DH) that can also be utilized since these homozygous populations can allow replicated and repeated experiments (Collard & Mackill, 2008). Downstream applications of QTL mapping ranges from understanding the genetic control of salinity tolerance mechanism, development of varieties with improved salinity tolerance, and the possibility of transferring the identified QTLs into a susceptible high yielding variety through cloning and transformation (Gregorio et al., 2002; Ismail et al., 2007; Thomson et al., 2010). Several studies in salt tolerance have identified QTLs associated with rice. Singh et al. (2007) identified seven QTLs for seedling traits related to salt stress and were mapped to five different chromosomes. Zhang et al. (1995) detected a major gene for salt tolerance and was mapped on chromosome 7, using an F_2 population derived from a cross between a sensitive original variety (77-170) and salt-tolerant japonica rice mutant (M-20). Mondal et al. (2019) identified 11 QTLs associated with reproductive stage salinity tolerance in chromosomes 1, 2, 5 and 11. The QTLs named *qSKC1* for shoot K^+ concentration was reported in chromosome 1 was reported for shoot K^+ concentration. It was located between 9.82 and13.30 Mb region (Koyama et al., 2001; Lin et al., 2004; Thomson et al., 2010; Wang et al., 2012) from which an HKT1;5 gene was cloned from Nona Bokra for salinity tolerance (Ren et al., 2005). A major QTL (Saltol) was identified in chromosome 1 for salt tolerance using F_8 recombinant inbred lines of IR29/Pokkali cross (Gregorio, 1997). The Saltol QTL

was described to be responsible for the Na^+-K^+uptake ratio and accounted for 64.3 to 80.2 % of the phenotypic variation in salt tolerance (Ismail & Bennett, 2004). RM8094 and RM10745 SSR markers could be useful for marker-assisted selection of Saltol QTL (Mohammadi-Nejad et al., 2010).

2.12. Genome Editing

Genome editing is a way of modifying DNA of a cell or organism with a very specific change. The CRISPR/Cas 9 system is an RNA-guided gene-editing tool with the fusion of Cas 9 endonuclease that targets DNA sequence of 5"-N20-NGG-3" where NGG is the PAM site for recognition of the target for cleaving (Xie et al., 2015). Besides Cas9, Cas 13 is also an important effector protein/antiviral against three diverse ssRNA in cell culture (Freije et al., 2019). Overall, because of its simplicity and high efficiency, the CRISPR/Cas9 technique is becoming popular to modify the gene for greater objectives crop improvement for food security. The most common and popular form of CRISPR/Cas is the type II CRISPR/Cas system because of their processing system of crRNA involves introduction of tracrRNA which is complementary to the repeat sequence in pre-crRNA. As a result, double stranded break is initiated by RNaseIII enzyme in the presence of Cas 9 endonuclease. Cas 9 has two domains; HNH and RUV-C which cleave the complementary and non-complementary strands of target DNA respectively (Jinek et al., 2012). Overall, because of its simplicity and high efficiency, CRISPR/Cas9 technique is becoming popular to modify gene for greater impact towards crop improvement for food security.

2.13. How CRISPR/Cas9 Works

The CRISPR/Cas9 system takes the place of zinc finger nuclease (ZFN) and TALEN because it is the simplest technique (Bao et al., 2019) as these two require protein engineering. For CRISPR/Cas9, single-stranded gRNA(sgRNA) is required to target the DNA region that is easy to construct by different online tools like CRISPR direct, CRISPR-P, etc. It occurs in three phases; (1) adaptive phase, where bacteria and archaea protects more than one CRISPR loci by integrating short fragments of foreign sequence known as protospacer adjacent motif (PAM) into the host, (2) in the expression phase CRISPR array is transcribed to produce pre-crRNA which is cleaved by enzymes to form short crRNA and lastly (3) in the interference phase crRNA guides Cas9 proteins to cleave the complementary plasmid or virus target sequences that match the spacers (Makarova et al., 2011). After inducing the double-stranded break by CRISPR/Cas9, they are repaired by non-homologous end joining (NHEJ) or by homologous recombination (HR). NHEJ is indefinite and creates gene knock-out mutations consisting mostly of insertions, deletions or sometimes frameshift mutations due to imprecise DNA repair, whereas HR is defined and leads to gene knock-in or gene replacement, with the presence of a donor DNA molecule (Figure 2.1).

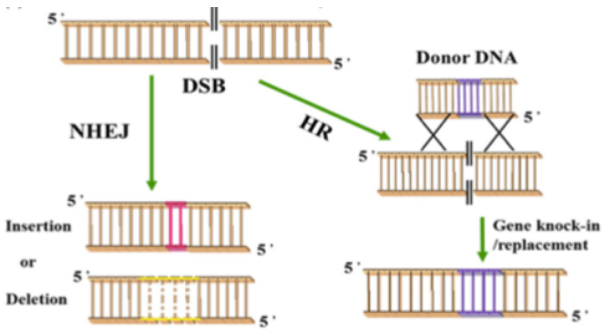

Figure 2.1. Repair mechanisms after double stranded break (adapted from Bao et al., 2018).

2.14. Problems Associated with CRISPR/Cas9 and Possible Solutions

One of the few discrepancies of the CRISPR/Cas9 technology is the relatively high frequency of off-target mutations reported in some of the earlier studies (Cong et al., 2013; Fu et al., 2013). Although initially all 20 nt sequence in the gRNA was considered to be more specific for target site recognition and cleavage, it was later shown that mismatches outside the seed region (8-12 nt at the 3′ end) are still tolerable (Cong et al., 2013; Jinek et al., 2012) depending on the total number and arrangement (Fu et al., 2013; Hsu et al., 2013). But every problem has some reasonable solution and to solve the off-target issues, several online tools have been developed based on the comparative analysis of 700 gRNAs (Hsu et al., 2013). Optimizing nuclease expression is another way to control specificity because high concentrations of gRNA and Cas9 can promote off-target effects (Pattanayak et al., 2013). So, the basic strategy to avoid off-target effects is the design of a specific gRNA by checking for the presence of homologous sequences in the genome. Another strategy is to use of a pair of Cas 9 nickase pair that creates single-strand nicks and produces a staggered DSB (Bortesi & Fischer, 2015) by mutating any one of the domains in Cas 9, so that Cas9 can cleave one strand of target DNA depending which one is mutated. For example, if D10A mutate the Ruv-C domain, then Cas 9 will cleave the complementary strand.

2.15. Construction of CRISPR/Cas9 System

Researchers have successfully adopted CRISPR/Cas9 to edit target genes in different plants. The fundamental elements for conducting CRISPR Cas9 experiments are Cas9 and sgRNA. But the successful construction of CRISPR/Cas9 with high editing efficiency depends on some factors like the expression of level of Cas9 and sgRNA, GC content of the

target DNA and sgRNA, codons of Cas9 (Bao et al., 2019). Generally, sgRNA and Cas9 expression cassettes can be introduced into the plant via Agrobacterium- mediated transformation. This expression of sgRNA and Cas9 is driven by some promotors, like U3/U6 and ubiquitin/cauliflower mosaic virus 35 S, respectively (Bao et al., 2019; Ma et al., 2015). Besides, Cas9 and sgRNA together form a synthetic complex called ribonucleoprotein (RNP), which could be delivered directly to the plant in the form of calli or shoot apical meristem (SAM) by particle bombardment.

2.16. Delivery Techniques and Screening of Mutant Plants

Three popular delivery techniques exist for plant gene editing. They are (1) Agrobacterium-mediated transformation (AMT), (2) biolistic, and (3) protoplast transformation (PT) (Baltes et al., 2017). Among them, AMT is popularly used for stable gene transformation.

After obtaining transformed progeny, it is necessary to verify either they are mutants or not. The methods for CRISPR/Cas 9 induced mutant screening are: restriction enzyme (RE) assay, surveyor nuclease and T7E1 assay and high-resolution melting analysis (HRMA) (Bao et al., 2019). When a mutant plant has a visible knock out phenotype, screening could be done quickly. For example, knock-out of the PDS gene results in albino phenotype, so the PDS gene is used as a selectable marker for plants (e.g., tobacco and rice). Generally, the transgene-free mutant plant could be achieved within 2 to 3 generations through segregation (Gao et al., 2016).

2.17. The Procedure of Conducting CRISPR/Cas9 Experiments in Agriculture

The procedures of agricultural experiments with CRISPR/Cas9 involve: (1) selection of the plant and target gene e.g., rice HKT gene, (2) selection of approach (knock-out, knock-in, large deletion, allele replacement), (3) target region selection (e.g., 1st exon), (4) designing gRNA (20nt+3 PAM), (5) produce construct or binary vector with Cas9 and gRNA expression cassettes (e.g. PRGEB32) or Cas9-gRNA complex (RNP), (6) transformation of a binary vector into the plant via AMT and biolistic delivery and RNP via biolistic and (7) screening of mutant plants by PCR/RE assay.

2.18. Successful Examples of Gene Editing in Plants

Although CRISPR/Cas9 is a newly emerging technique in crop improvement, it has proven its value in several areas: (1) increasing yield: in order to increase yield in rice, knock-out of GW2, GW5 and TGW6 increase grain size and weight around 20% to 50% than the wild type (Xu et al., 2016). Knock-out of flowering repressor SP5G gene exhibits day length insensitive tomato; thus, early maturity occurs due to rapid flowering. (2) Improving biotic and abiotic resistance: deletion of OsSWEET13 promotor leads to BLB resistance in rice (Zhou et al., 2015) and knock-in GOS 2 promotor in place of ARGOS8 via HDR increased drought resistance and yield in maize (Shi et al., 2017). (3) Increasing quality: knock-out of GBSS in potato, reduce amylose content and increase amylopectin (Andersson et al., 2017) and (4) nutritional improvement: simultaneous knockout of FAD2-1A and FAD2-1B significantly increase oleic acid in canola (Haun et al., 2014).

2.19. Beyond Gene Editing

CRISPR/Cas9 is an ideal gene regulation tool (Bao et al., 2019). By introducing mutations in both nuclease domains of Cas9, deactivated Cas9 (dCas9) is created which acts as a gene regulator. The dCas9 can be fused with transcriptional activator or repressor to perturb gene expression, such as fusion with VP64 (transcriptional activator) to increase transcriptional activity and dCas9-KRAB, which recruits chromatin-modifying complexes, to more efficiently silence the gene transcription (Seth & Harish, 2016). Moreover, CRISPRa up-regulates the transcription of endogenous genes and is used for overexpression of the desired gene to obtain high yield varieties and CRISPRi uses to generate mutants with down-regulated expression of selected genes.

As conventional breeding takes enormous time and labor, genome editing is a powerful tool to change the world's future agricultural scenario. Already some success has been achieved in gene editing of some major cereal and a few horticultural crops. Since, food production needs to be increased for future food security, CRISPR/Cas 9 technology may be a milestone to achieve that. The major concern for all genome editing approaches is successful regeneration, which is really challenging as few crops are responsive to regenerate even after successful transformation. To overcome this problem, (Lowe et al., 2016) describes baby boom (Bbm) and Wuschel2 (Wus 2) transcription factor for the transformation by AMT and biolistic with higher efficiency (15% to 85%). Besides, *in planta* transformation is also fruitful for regeneration (Saifi et al., 2020). Therefore, reducing or eliminating these constraints gets us closer to the ultimate goal of genome editing in agriculture.

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3. MAPPING QTLs FOR REPRODUCTIVE STAGE SALINITY TOLERANCE IN RICE USING BC_1F_2 POPULATION OF CSR28 x BRRI dhan28

3.1. Introduction

Rice (*Oryza sativa L.*) is a major staple food for almost half of the world's population; sustainable rice production is essential for the world's food security. Unfavorable environmental conditions such as salinity, drought, heat, and submergence pose a huge threat to agricultural production and productivity and challenge future food security. Salinity is one of the serious environmental stresses that limit the productivity of agricultural crops, which are most sensitive to the presence of high concentrations of salts in the soil (Flowers and Yeo 1995, Flowers, 2004). The mechanism of salinity tolerance is still not very clear, but tolerant plants use a combination of mechanisms to overcome salinity stress. Salinity stress inhibits plant growth through many disruptions, such as osmotic effects, excessive uptake of toxic ions (Na^+ and Cl^-); and poor regulation of antioxidants with impaired signal pathways (Munns 2002, Singh and Flowers 2020).

Rice is relatively tolerant of salt stress during germination, active tillering and grain filling and is sensitive during the early seedling and reproductive stages (Singh et al. 2007, Singh and Flowers 2020). Yeo and Flowers. (1986) mentioned that during reproductive-stage development, tolerant genotypes tended to exclude salt, thus resulting in less salt concentration in flag leaves and developing panicles, which resulted in higher grain yield. Rao et al. (2008) reported a grain yield reduction of 27% to 50% when salinity stress of ECe of 8 dS/m was imposed on different rice cultivars.

The effect of salinity on rice depends on the amount of salt, duration of exposure, cultivar, crop growth stage, water regime, soil physical properties, temperature and solar radiation (Neue et al. 1998, Ali et al. 2013). Higher salinity at the reproductive stage significantly reduces spikelets per panicle, tiller number, fertility, panicle length and the number of primary branches per panicle (Heenan et al. 1988, Cui et al. 1995, Khatun et al. 1995, Zeng and Shannon 2000, Zeng et al. 2002, Singh et al. 2010). The number of spikelets per panicle was found to be the most sensitive yield component. This component is determined at the early reproductive stage, around panicle initiation (Counce et al. 2000).

Although salinity at the reproductive stage reduces grain yield much more than the vegetative stage but there have been only very few studies in rice for salinity tolerance at the reproductive stage due to the difficulty of achieving reliable stage-specific phenotyping techniques (Ahmadizadeh et al., 2016). Further, the salinity rice breeding progress is slower because of genetic complexity and environmental factors, as salinity tolerance is governed by many genes that exhibit the polygenic nature. The genetics behind salinity tolerance can be revealed by using quantitative trait locus (QTLs) analysis (Gimhani et al. 2016; Negrão et al. 2011). Recent advances in molecular marker technology have enabled the dissection of the molecular mechanisms of salinity tolerance to identify major-effect QTLs (Munns 2005; Tuberosa and Salvi 2006; Passioura et al. 2007; Thomson et al. 2010, 2012; Thomson 2014; Hossain et al. 2015). Single nucleotide polymorphisms (SNPs) are the markers of choice for most high throughput genotyping applications because they are ubiquitous in eukaryotic genomes, cost-effective to assay using automated platforms, and biallelic in nature, which is useful for allele calling, data analysis and data-basing (Thomson et al., 2017).

In addition, salt-affected areas are expected to increase due to the adverse effect of climate change and sea-level rise (IPCC, 2019). Therefore, improving the productivity of salt-stressed areas is considered essential to meet the increasing food demand of the growing population. Thus, this chapter discusses about the use of a mapping population derived from the two popular indica rice varieties to identify appropriate QTLs for salinity tolerance at the reproductive stage of rice.

CSR28 is an Indian semi-dwarf landrace with long slender grains and tolerant to salinity-stress at the reproductive stage of rice (Mohammadi, et al. 2014). CSR28 also had the highest positive general combining ability (GCA) values for K^+ uptake. Therefore, this landrace could be used to improve the K^+ uptake of breeding lines to develop better and more tolerant rice varieties. Like CSR28, BRRI dhan28, a Bangladeshi indica rice mega variety, has desirable characteristics for higher productivity, except the variety is highly sensitive to salinity stress. BRRI dhan28 was developed by the Bangladesh Rice Research Institute (BRRI) and released by the Government of Bangladesh in 1994. Since then, BRRI dhan28 has shown its potential to adapt in diverse favorable agro-ecological zones and popularity among the diverse consumers. In this study, the BC_1F_2 mapping population developed from the cross between CSR28 and BRRI dhan28 at the International Rice Research Institute (IRRI) for identifying QTLs associated with salinity related traits.

3.2. Objective

The general objective of this study was to identify quantitative trait loci (QTLs) for salinity tolerance at the reproductive stage of rice plants using the BC_1F_2 population derived from a salt-tolerant Indian variety, CSR28 and a salt-sensitive Bangladeshi variety, BRRI dhan28. The specific objectives were:

a. To screen the BC_1F_2 population, along with their parents under salinity stress for establishing the relative importance of different traits associated with the reproductive stage salinity tolerance in rice.

b. To genotype the BC_1F_2 population for mapping the large effect QTLs responsible for tolerance to salt-stress at the reproductive stage of rice.

c. To identify candidate diagnostic markers linked to the QTLs conferring salinity tolerance from the tolerant parents.

d. To identify the candidate genes.

3.3. Materials and Methods

3.3.1. Study Site

The study was conducted at two locations – (i) the phenotypic screening and physiological characterization were conducted at the International Rice Research Institute (IRRI, 14.16774°N and 121.254547°E), Los Baños, Philippines from February to June 2018; while (ii) the molecular characterization was done at the Agri Genomics Laboratory (AGL), Genomics and Bioinformatics Services (GBS) and Crop Genome Editing Laboratory (CGEL) of Texas A&M University (TAMU, 30.6185° N, 96.3365° W), College Station, USA from January 2019 to May 2020.

3.3.2. Plant Materials

A salt-tolerant Indian variety, CSR28 and a salt-sensitive Bangladeshi rice variety, BRRI dhan28 was used as the parents to develop a mapping population. The BC_1F_2 QTL mapping populations was generated by crossing the salt-tolerant donor parents (CSR28) with the salt-sensitive parent (BRRI dhan28). All the parents belong to sub-species Indica. The cross, CSR28 x BRRI dhan28were developed at the rice breeding platform of the International Rice Research Institute (IRRI) crossing program to generate F_1 and then backcross to recurrent parent, BRRI dhan28 to produce the BC_1F_2 progenies (IBP, www.integratedbreeding.net). Parents for each population were used as the checks for the respective population.

3.3.3. Experimental Design and Set-up

The phenotypic experiments with desired (EC 10 dS/m) salinity level and a control treatment were set up at IRRI in Completely Randomized Design (CRD). Screening of BC_1F_2 population derived from the crosses between CSR28 x BRRI dhan28 was done in the 2018 dry season (February-June). The genotypic experiment was conducted at the TAMU (AGL, GBS and CGEL Labs).

In the phenotypic experiments, a set of 624 young seedlings or individuals derived from the crosses between CSR28 x BRRI dhan28 was sown in pots and those pots were transferred into three large concrete tanks having a dimension of 680 cm x 105 cm x 22 cm (length x width x depth). The concrete tanks were filled-up with ordinary tap water until putting salt (NaCl) at the reproductive stage. These 624 individuals, 435 in saline treatment and 189 in normal water treatment (control), were used for phenotyping under natural environment except they had rain shelters placed at about 1.5 m above the ground to protect the saline treatments from rains at the reproductive stage only (Fig. 3.1).

a. Vegetative Stage

b. Reproductive Stage

Figure 3.1. Experimental sites at the International Rice Research Institute (IRRI), Philippines (a) at vegetative stage and (b) at reproductive stage of rice plant.

3.3.4. Phenotyping of Parents and BC_1F_2 Individuals

3.3.4.1. Seedlings Preparation and Management

Seeds of 624 BC_1F_2 individuals and parents were soaked and put into the oven for three to four days at 30^oC to break dormancy for germination. After that, the seeds were randomly sown manually in the small trays and allowed to grow for 25 days. During the growth period of the rice seedlings, regular watering and foliar spraying was done. The solution for foliar spray was prepared with 2.5 gm of urea, 1.5 gm of $FeSO_4$ and 5 to 6 drops of liquid soap per liter of water (Ahmadizadeh et al., 2016; Singh et al., 2008) and sprayed four times in both experiments during second and third week of the seedlings when the leaves turned yellowish.

3.3.4.2. Pot Preparation and Fertilizer Application

Twenty-five days old seedlings of rice plant were transplanted manually in the perforated cylindrical pots. The dimension of the perforated pot was 15 cm in height and 11 cm in diameter with 3-4 mm holes spaced 2 cm apart. Each pot contained a plastic sieve bag filled with sterilized soil up to about 1 cm above the topmost circle of the holes, about 3 cm below the top of the cylinder. The sterilized soil was fertilized with 50, 25, and 25 mg of N, P, and K per kilogram of soil, respectively (Ahmadizadeh et al., 2016, Singh et al., 2008).

3.3.4.3. Transplanting and Management

Initially 2-3 seedlings were transplanted manually in each perforated plot; later on, extra plants were thinned out to keep one seedling per pot (Ahmadizadeh et al., 2016). After transplanting, the pots were placed in the concrete tank. The concrete tanks were filled with

normal tap water for growth and development of the rice plant. A water depth of 12-14 cm was maintained to keep the bottom 75% of the pots under water throughout the growing season of rice. All the plants were grown in normal water until the appearance of the first flag leaf and immediately transferred into the saline water after the plants encountered/reached at the booting stage.

Preventive spraying of Cymbush and Cartap was done at 15 days after transplanting to protect the rice plants from worm and maggot, which were commonly observed at IRRI (Ahmadizadeh et al., 2016). Besides, regular inspection on the attack of pest and diseases was observed. Cymbush and Cartapwere applied at about 15 days interval from transplanting to booting stage. There was no pest and diseases were observed when the plants were in the saline condition. But the normal treated plants were affected by pest and diseases and spraying was done at 15 days interval during reproductive stage to control it.

3.3.4.4. Salinity Treatment and Management

3.3.4.4.1. Leaf Pruning

Leaf pruning was done manually at the booting (gametophytic) stage of rice plant. Only flag leaf and penultimate leaves were kept in each plant, other leaves were removed or clipped. To make growth-stage dependent phenotyping, it is indispensable to precisely identify the stage during when salinity stress should be applied to plants. Indeed, the gametophytic stage is the appropriate stage when plants should be salinized. But salt takes a few days to reach to the panicle as it goes first to the oldest leaf sheath/leaves and then to the second oldest leaves/leaf sheath and so on in a sequential way (Ahmadizadeh et al., 2016). Therefore, due to the systematic sequential manner of reaching toxic ions (Na^+) from

the oldest leaves to relatively younger leaves and ultimately to the flag leaf and inflorescence (i.e. panicle), applying salt at booting stage would definitely delay in salt transporting into the reproductive organs because of the sink. As a result, plants would escape the real stress at the booting stage. To solve these problems and to direct the salt movement to the reproductive organs quickly and efficiently; older leaves of the plants were pruned and kept only the flag leaf and penultimate leaf (Fig. 3.2). In this case, salt would have no scope to compartmentalize and consequently it will reach directly to the reproductive organ and express their real signs and symptoms after having saline stress.

Immediately after identifying the first flag leaf, leaf pruning was done and the first flag leaf was tagged with a proper label containing date of booting or first flag leaf appearance, date of removing from saline water as well as date of flag leaf collection and the serial number of plants.

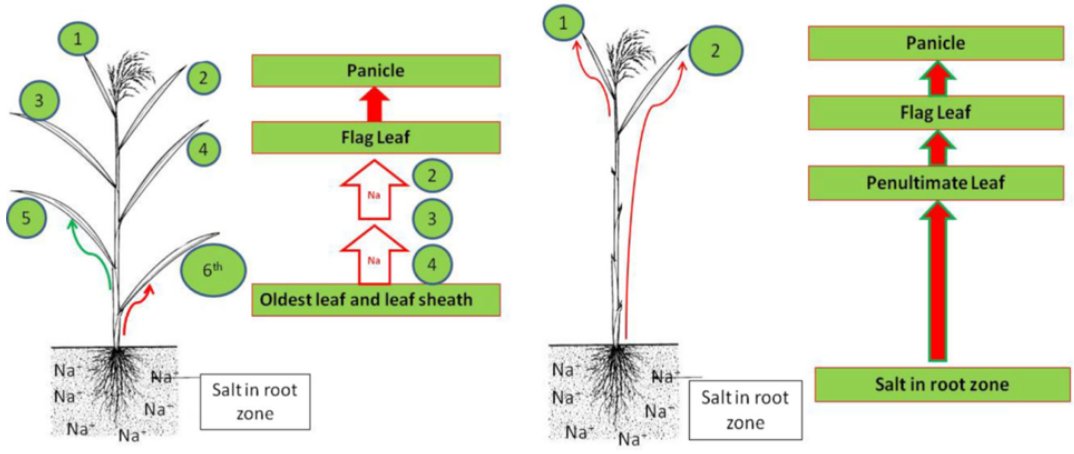


Figure 3.2. Leaf pruning and a schematic diagram of salt uptake mechanism in rice plant (adapted from Ahmadizadeh et al., 2016).

3.3.4.4.2. Salinity Application at Booting Stage

Saline solution was prepared by dissolving NaCl into the tap water and raised up to EC 10 dS/m. Salinization was done just after the appearance of the first flag leaf i.e. when plant entered into the booting stage (Ahmadizadeh et al., 2016). The plants were checked every day to identify occurrence of the booting stage in each plant. As this mapping population was BC_1F_2, the response of every individual was different because of segregation; that means not every plant showed the booting stage at the same time as the duration of vegetative stage varies from plant to plant (Fig. 3.3).

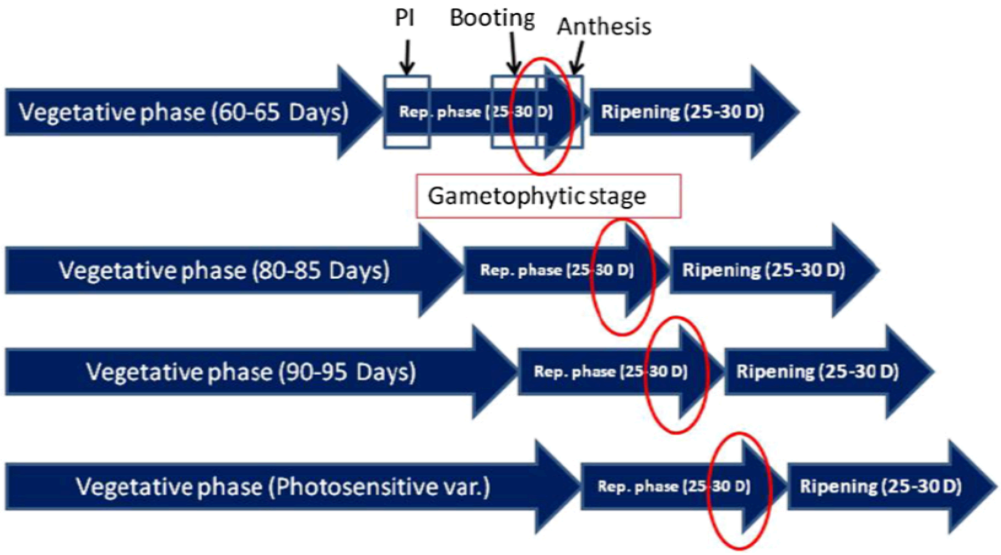


Figure 3.3. Variation in vegetative growth stages in rice (adapted from Ahmadizadeh et al., 2016).

After pruning and tagging, a total of 435 plants were transferred into saline water of EC 10 dS/m and the rest of the 189 plants were kept under normal water, which was checked daily and maintained the constant EC for continuously 20 days from first day of booting. After 20 days of salinity treatment, the plants were shifted back to the normal water and the first flag leaf was collected immediately, which was marked or tagged to determine the Na^+ and K^+ ion concentration of the leaf samples.

3.3.5. Determination of Na and K Ion Concentration in Flag Leaf

3.3.5.1. Flag Leaf Collection and Preparation

The flag leaves were collected manually to measure sodium (Na) and potassium (K) ion concentration; i.e. percentage of Na^+ and K^+, and the ratio of Na^+ and K^+. As the flag leaf was quite big to place in a falcon tube; each flag leaf was cut into small portions i.e. in lengthwise from the base, lamina, and tip. But when it was sampled from the top, middle, or bottom portion; the ion concentration may vary due to ionic influx variation within the leaf (Palao et al., 2013). After cutting, washing of the flag leaf samples was done two times with Reverse Osmosis (RO) water and one time with nanopure water. Then leaf samples were tap and dried down with the paper towel to remove excess water and put it into the 10 ml tube which was filled out with 0.1 N acetic acid solutions for digestion.

3.3.5.2. Preparation of 0.1 N Acetic Acid Solutions and Digestion

To make 1 liter of 0.1N acetic acid solution, 994 ml of nanopure water was mixed with 6 ml of glacial acetic acid (GAA) (Ahmadizadeh et al., 2016). To determine Na^+ and K^+ concentration, the leaf samples were placed in screw-capped bottles or falcon tubes filled

with 10 ml of 0.1N acetic acid solution. The leaf samples were digested into the water bath at 90°C for 2 hours. The samples were taken out from the stock solution and oven dried for 5 days at 50^0C. Then the dry weight of the flag leaf samples was taken.

3.3.5.3. Concentration of Na^+ and K^+

To analyze the ion concentration, stock solution was diluted 10 times (1ml of stock solution and 9 ml of nanopore water) and Perkin-Elmer Analyst 300 atomic absorption spectrophotometer was used to analyze Na^+ and K^+ concentration (Ahmadizadeh et al., 2016). Appropriate standards were prepared to maintain the accuracy of the results. The concentration of the standards was 0, 2, 4, 6, 8 and 10 ppm. The Na and K ion concentration was calculated by using the formula:

Na or K = [C* (d*V/1000)]/dwt]

Where,

Na or K = Concentration of sodium and potassium ion (mmol/g dwt)

C = Concentration of sample aliquot based on atomic absorption spectrophotometer reading as determined relative to standard curve

d = dilution factor

V = extraction volume (ml)

dwt = oven dry weight of the plant leaf (g)

3.3.6. Estimation of Yield and Agronomic Parameters

Data on grain yield, yield components and important agronomic parameters such as plant height, number of productive and unproductive tillers, panicle number, panicle length, number of filled and unfilled spikelets and grain yield per plant was collected from 624 plants manually at maturity.

3.3.6.1. Plant Height

Plant height of all the plants was measured from soil surface to the tip of the tallest panicle (awns excluded). The height of the plants was taken at the time of harvest.

3.3.6.2. Productive Tillers

The number of productive tillers per plant was counted manually at the time of harvest. But dried tillers and those that have undergone senescence was excluded from the count.

3.3.6.3. Panicle Length

Panicles of the plants were collected at harvest and the total number of panicles per plant was determined. The panicles which did not bear even a single grain were excluded from the count. Three panicles from each plant were selected randomly and the length was measured from pulvinus to the tip of the topmost grain.

3.3.6.4. Filled and Unfilled Spikelets

Immediately after measuring of the panicle length, filled and unfilled grains were manually removed from the panicles and number of filled and unfilled spikelets per plant was recorded. Then the percent filled spikelets were determined by dividing the number of filled and unfilled spikelets to the total spikelets of each plant. And when the percent unfilled spikelets were used, it was calculated as 100 - % filled spikelets.

3.3.6.5. Grain Yield

Grain yield was taken at harvest. After threshing and cleaning, weight of the dried filled grains per plant was measured. The yield of rice was expressed in gm per plant after drying.

3.3.7. Salinity Scoring

Salinity scoring was done to identify the tolerant and sensitive plants based on visual symptoms just after the harvest by using Modified Standard Evaluation System (SES) developed at IRRI (Mondal et al., 2016, 2019) (Table 3.1). The procedure used in the phenotypic evaluation of salinity tolerance was described in the Standard Evaluation System Manual (IRRI, 2013).

Table 3.1. Modified standard evaluation system (SES) for scoring of visual salt injury at reproductive stage in rice (adapted from IRRI, 2013).

Score	Observation	Rating
1	Normal growth, spikelet sterility at <5%	Highly tolerant
3	Growth slightly stunted, spikelet sterility at 5%-20%	Tolerant
5	Growth moderately stunted, one-fourth (25%) of all leaves brown, panicles partially exerted, spikelet sterility at 21%-40%	Moderately tolerant
7	Growth severely stunted with about half (50%) of all the leaves become brown, panicle poorly exerted, high sterility at 41%-70%	Sensitive
9	Growth severely stunted with about of all the leaves become brown, panicle not exerted, high sterility at >70%	Highly sensitive

3.3.8. Genotyping of Parents and BC_1F_2 Progenies

3.3.8.1. DNA Extraction

Out of 435 BC_1F_2 populations, 192 individuals were selected for genotyping. These genotypes were selected by selective genotyping (choosing most tolerant and sensitive individuals) from the mapping population based on the visual salinity score (SES) and grain yield per plant. From each plant, 25 days old leaves were collected and lyophilized for long time storage (Ahmadizadeh et al., 2016). Then genomic DNA was extracted from those leaf samples using a standard CTAB method (Doyle, 1991b). After DNA extraction, DNA quality was checked by agarose gel electrophoresis and quantity was measured by nanodrop.

3.3.8.2. Kompetitive Allele Specific PCR (KASP) Genotyping

Kompetitive Allele Specific PCR (KASP) genotyping assays are based on competitive allele-specific PCR and enable biallelic scoring of single nucleotide

polymorphisms (SNPs) and insertions and deletions (Indels) at specific loci (LGC, 2013). The KASP genotyping was done by the mixing of the SNP-specific KASP assay, universal KASP master mix and DNA samples into a PCR plate. Then thermal cycling reaction of the KASP mixture was performed followed by an end-point fluorescent read (LGC, 2013). The KASP genotyping system is composed of two components: (i) the SNP specific KASP assay mix; which was separately purchased as a KASP by Design (KBD) that contains two allele specific forward primers that differ at their 3/ end and one common reverse primer and (ii) the universal master mix which contains FAM and HEX specific FRET (fluorescence resonant energy transfer) cassette and Taq polymerase with optimized buffer (LGC 2013-KASP Genotyping Chemistry User Guide and Manual, LGC). Unique tail sequence of each of the allele specific primers corresponds with a universal FRET cassette; one labelled with FAM™ dye and another one with HEX™ dye. KASP master mix contains ROX as a passive reference dye for well normalization.

For QTL analysis of 192 individuals from CSR28 x BRRI dhan28, a total of 230 markers were selected from the integrated breeding platform based on their polymorphism (IBP, www.integratedbreeding.net). Initially 160 markers were sent to the LGC Biosearch Technology (LGC, 2019) to convert those into KASP marker. After mixing of the two aforesaid components into a PCR plate (Table 3.2), the KASP chemistry used water bath based thermal cycler (Hydrocycler^{TM}) to perform PCR steps. An S1000 Thermal Cycler (Bio-Rad) was used for KASP genotyping with following conditions (Table 3.3).

Table 3.2. Constituent reagent volumes for making KASP genotyping mix. *DNA samples diluted to final concentration of 5 - 50 ng per reaction (adapted from LGC, 2013).

Components	KASP genotyping mix assembly			
	Wet DNA method (µL)		Dry DNA method (µL)	
DNA*	2.5	5	N/A	N/A
2x Master mix	2.5	5	2.5	5
Primer mix	0.07	0.14	0.07	0.14
H2O	N/A	N/A	2.5	5
Total reaction volume	5	10	5	10

Table 3.3. Thermal cycling condition for KASP chemistry (adapted from LGC, 2013).

94 ^{0}C for 15 minutes	Hot-start activation
94 ^{0}C for 20 seconds 61-55 ^{0}C for 60 seconds (dropping 0.6 ^{0}C per cycle)	10 cycles
94 ^{0}C for 20 s 55 ^{0}C for 60 seconds	26 cycles

After completion of PCR steps, fluorescence intensity was visualized with a microplate reader (PHERAstar^{plus}, BMG LABTECH, Germany) and analyzed by Kluster Caller software 7 (LGC, 2014). A homozygous sample for the allele reported by FAM only generates FAM fluorescence during the KASP reaction which will be plotted close to the X-axis, that represents high FAM signal and no HEX signal (Fig. 3.4-blue points). While a homozygous sample for the allele reported by HEX only generates HEX fluorescence and data point will be plotted close to the Y axis, representing high HEX signal and no FAM signal (Fig. 3.4-red points). Lastly the heterozygous sample will be plotted in the center of the plot that contain both FAM and HEX alleles (Fig. 3.4-green points). To ensure the reliability of the results, a KASP reaction without any template DNA must be included as a

negative control. This is typically referred to as a no template control or NTC. The NTC will not generate any fluorescence and the data point will therefore be plotted at the origin (black data points in Fig. 3.4).

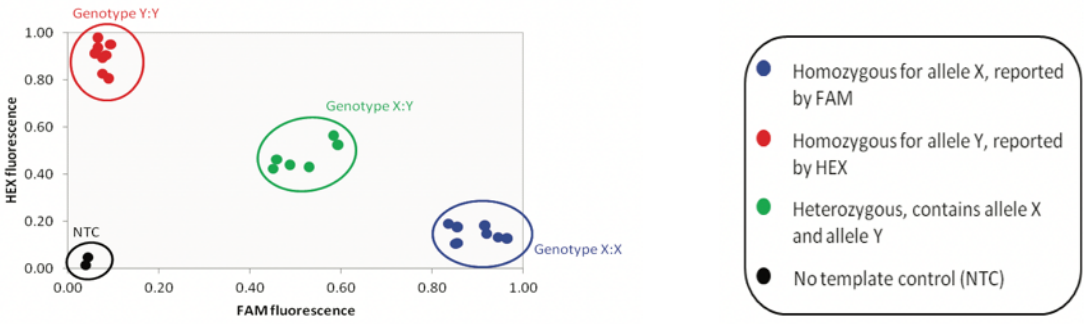


Figure 3.4. Genotyping cluster plot and each data point represent the fluorescence signal of individual DNA sample (adapted from LGC, 2013).

Not all of the 160 markers showed polymorphism (LGC, 2014). That is why initially polymorphism survey was done to check the status of the markers i.e. if the markers were polymorphic or not. Out of 160 markers, 82 markers showed polymorphism and rest of the markers were monomorphic. Monomorphic markers were discarded from the analysis and those 82 markers were used for SNP genotyping. Each polymorphic marker was used to run with all 192 samples to identify the SNPs.

Other than ordering markers from LGC, an additional 70 primers were designed by using IDT protocols (You et al., 2008). These markers were also selected from the Integrated Breeding Platform (IBP) based on their polymorphism and marker positions after the monomorphic markers were discarded previously. Then similar PCR steps described on Table 3.3 were followed to analyze the marker status either polymorphic or monomorphic. For the primer design, BatchPrimer3 V1.0 software was used which was a high-throughput web tool for picking PCR and sequencing primers (LGC, 2014). Instead of picking normal

generic primer, allele specific and allele flanking primers were selected as type of primers in BatchPrimer3 V1.0. After designing primers, allele-flanking type was chosen depending on the direction of allele-specific types. In case of reverse allele-specific types, forward allele-flanking type was chosen and vice versa. Letter 'C' was added at the end of the primer names for flanking types primers and Letter 'X' and 'Y' was added for each of the allele-specific pair. After having all of the sequences for both flanking and allele-specific type primers, 'tail' sequences were added to allele-specific primers where the fluorescence was attached. Those two sequences were GAAGGTGACCAAGTTCATGCT for FAM (X) and GAAGGTCGGAGTCAACGGATT for VIC (Y).

3.3.8.3. Linkage Map Construction and QTL Mapping for Salinity Tolerance

Linkage Map was constructed using the IciMapping software version 4.2 (www.isbreeding.net/software/?type=detail&id=28) based on recombination frequency. In this study, SNP ordering method was used to ordering algorithm of RECORD as proposed by (van Os et al., 2005). After SNP ordering, rippling was done with COUNT algorithm for fine tuning of the linkage map.

In addition, QTL mapping was done using IciMapping software version 4.2 which is an extensible, interactive software for mapping quantitative trait loci (QTLs). To identify precise QTLs for salinity tolerance, Inclusive composite interval mapping (ICIM-ADD) was used. This program was used to determine the association between individual marker loci and QTLs. It also uses marker-genotype groups as class variables for the detection of linkage between markers and putative QTLs. The critical threshold value for QTL detection was calculated by 1000 random permutations of the phenotypic data to establish an experiment-

wise significance value at 0.05 as suggested by (Churchill & Doerge, 1994). This critical threshold value declared QTL at a 95 % level statistical confidence. Default LOD threshold score of 3.0 was also used and interval map distances based on the result of linkage map analysis were used to determine the association between markers and QTL. The proportion of the total phenotypic variation explained by each QTL was calculated as R^2 value (R^2 = PVE, phenotypic variation explained by the QTL) and additive effects were also determined for each trait. The QTLs were named using the procedure suggested by McCouch et al. (1997) and McCouch & CGSNL (2008).

3.3.9. Identification of Candidate Genes

Q-TARO database was used for identification of candidate genes of nearby quantitative trait locus (QTLs) identified in this study. The compiled gene information table was used for direct comparisons of the identified QTLs. In addition, QTL Genome Viewer was used to view the genomic location of identified QTLs as well as nearby candidate genes or QTLs within 200 kilobase (kb) region.

3.4. Results and Discussion

3.4.1 Responses of the Parental Lines and BC_1F_2 Progenies to Salt Stress

A large number of BC_1F_2 lines derived from the cross between a sensitive variety, BRRI dhan28 and a salt tolerant variety, CSR28 (CSR28 x BRRI dhan28) were evaluated under salt stress in the concrete tank at the International Rice Research Institute (IRRI), Philippines. Both the tolerant and sensitive parents were from the indica landraces. Salinity stress of EC 10 dS/m was applied at the reproductive stage (booting to maturity) of rice plant.

Total genotypes from the CSR28 x BRRI dhan28 were 624, of which salt stress was imposed on 435 individuals at the reproductive stage and 189 individuals were grown under no-stress (control) condition. Out of 435 individuals, 202 plants were classified as tolerant to moderately tolerant and 233 as sensitive to highly sensitive based on their SES score and grain yield (IRRI, 2013).

Selective genotyping method was used to identify the most tolerant and sensitive progenies. After screening based on grain yield and SES, the number of extremely tolerant and sensitive BC_1F_2 progenies from CSR28 x BRRI dhan28 were 98 and 92 respectively. The effect of salinity on agronomic and physiological characters is discussed in the subsequent sections below:

3.4.1.1. Plant Height

The plant height of the progenies of CSR28 x BRRI dhan28 varied from 12.0 to 166.5 cm under control condition and it varied from 11.5 to 130.5 cm under salinity stress condition (Table 3.4 and Fig.3.5). The plant height between control (no-stress) and salinity treatments varied significantly (Table.3.5, Fig. 3.6).

The interquartile range is explained the how the data is dispersed within the treatments. For the progenies of CSR28 x BRRI dhan28, the interquartile range of the control and salinity stress was similar (21.5 cm and 21.0 cm). But the median value of plant height of the control and stress treatments varied and they were 110.0 cm and 97.0 cm, respectively (Fig. 3.6). Among the families of CSR28 x BRRI dhan28, IR129282-20 had the highest plant height and IR129282-11 showed lowest plant height under both control and salinity stress (Fig. 3.7).

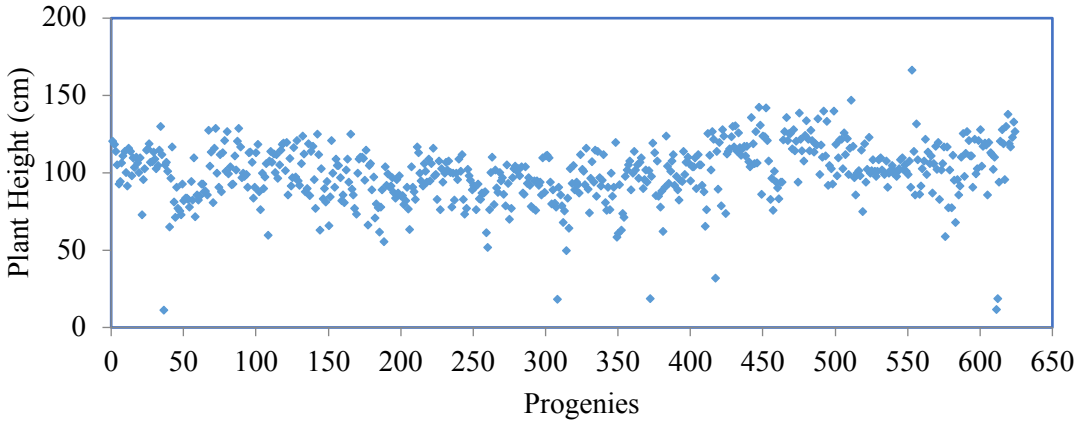
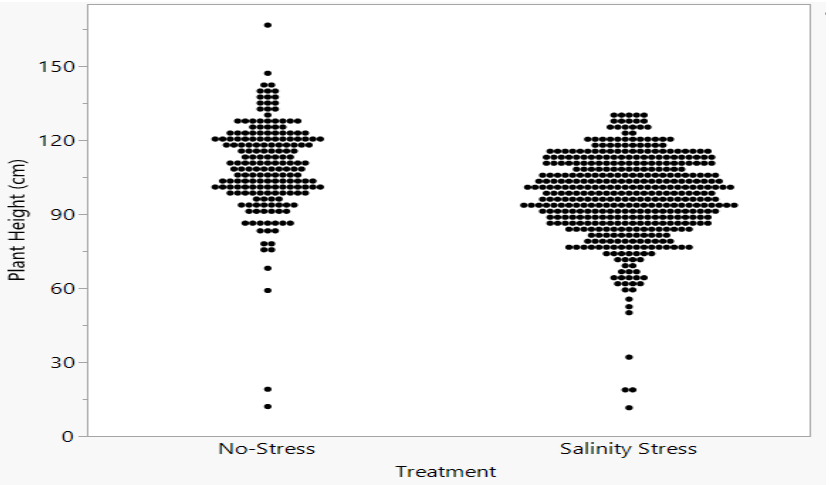


Figure 3.5. Scattered plot of plant height of all the BC_1F_2 progenies of rice from CSR28 x BRRI dhan28.

a. Scatter plot



b. Box plot

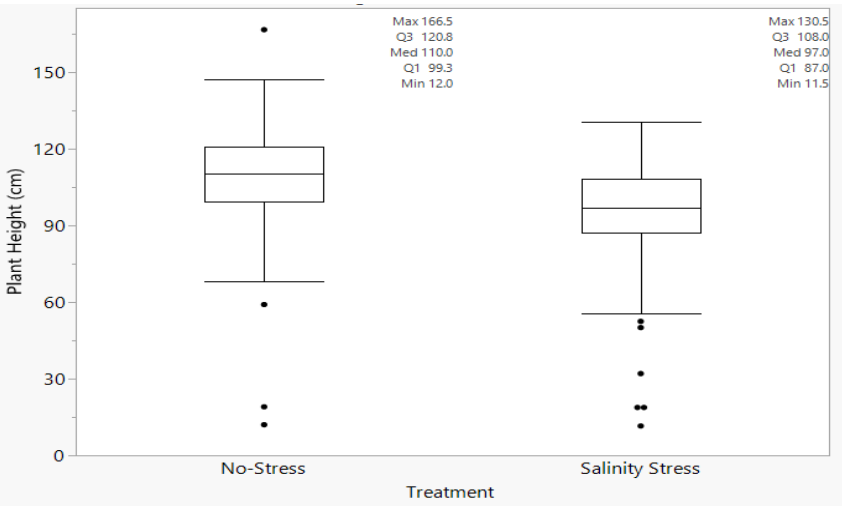


Figure 3.6. Scattered (a) and box (b) plots of the plant height of BC_1F_2 individuals under normal and salinity stress at reproductive stage of rice.

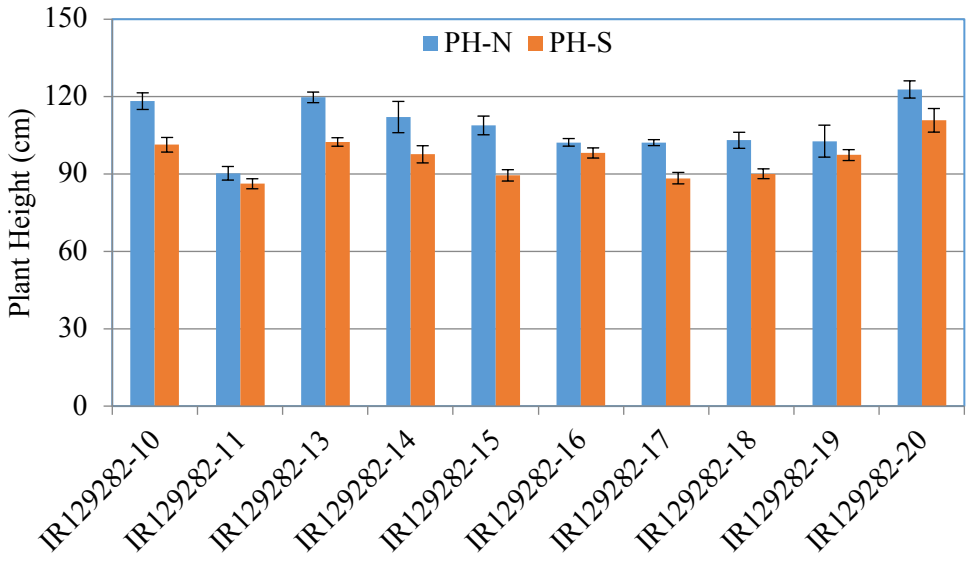


Figure 3.7. Mean plant height of the rice families of CSR28 x BRRI dhan28 progenies under control (PH-N) and salinity stress (PH-S). Vertical and capped bar indicates standard error of the mean plant height of 7 to 85 plants per family.

The plant height of the tolerant and sensitive parents was 122.4 cm and 90.0 cm, respectively under no-stress condition (Table 3.4, Fig. 3.8). In contrast, the mean plant height of the tolerant and sensitive progenies of CSR28 x BRRI dhan28 was105.5 cm and 84.9 cm, respectively under salinity stress and it was 108.8cm under no-stress condition. Considering selective genotyping, the plant height differed significantly between tolerant and sensitive individuals (Table 3.5, Fig. 3.8,). Reduction in plant height of sensitive genotypes was 12.3% over tolerant genotypes (Table 3.4)

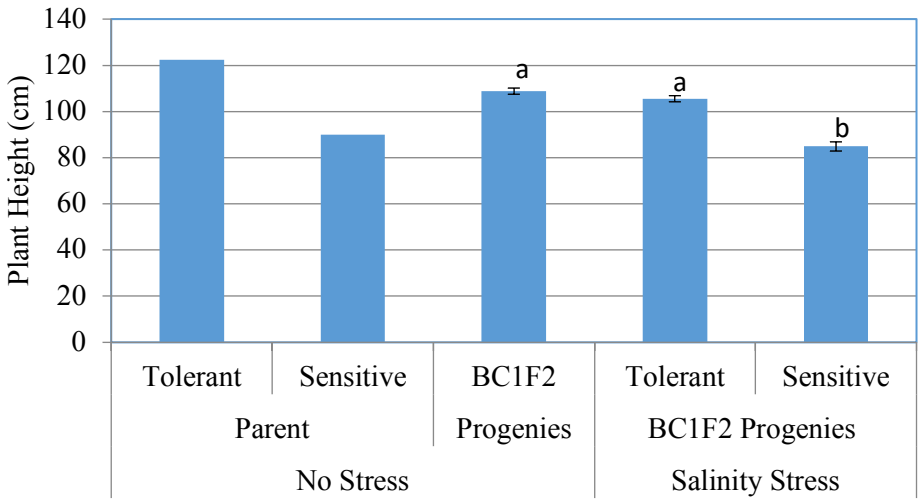


Figure 3.8. Mean plant height of tolerant and sensitive parents under control (no-stress), and their BC_1F_2 progenies under control and salinity stress at reproductive stage of rice. Vertical and capped bar indicates standard error of the mean plant height of 189 plants grown under no-stress, 98 tolerant and 92 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

Table 3.4. Descriptive statistics of the BC_1F_2 progenies of the cross, CSR28 x BRRI dhan28.

Traits	Parents		BC_1F_2 Progenies of CSR28 x BRRI dhan28							Decrease over No-Stress (%)		Decrease Sensitive over Tolerant (%)
	CSR 28	BRRI dhan28	Mean	Range	Skew-ness	SE	No-Stress	Tolerant	Sensitive	Tolerant	Sensitive	
Plant Height (cm)	122.4	90	96.39	11.5-130.5	-0.965	0.810	108.81	102.30	89.72	5.98	17.54	12.30
Productive Tiller (no/plant)	8	25	12.59	2-27	-0.183	0.215	16.48	14.18	10.57	13.95	35.86	25.46
Panicle Length (cm)	29	27	24.30	12.3-34.9	-0.297	0.147	26.03	25.07	23.06	3.68	11.40	8.01
Filled Spiketets (no/plant)	957	2349	265.34	0-1009	0.830	8.968	528.02	406.89	114.76	22.94	78.27	71.80
Unfilled Spiketets (no/plant)	451	617	738.95	76-2114	0.600	17.193	870.76	774.29	631.65	11.08	27.46	18.42
Filled Spikelets (%)	67.97	79.20	25.51	0-72.7	0.329	0.711	38.05	35.49	15.42	6.72	59.47	56.55
Grain Yield (g/plant)	17.15	54.98	5.74	0-25.1	0.959	0.202	14.01	11.80	1.52	15.77	89.15	87.12
Na-K Ratio	1.613	3.105	0.26	0.005-1.3	1.903	0.008		0.23	0.32			-39.26

Table 3.5. ANOVA of the BC_1F_2 genotypes of CSR28 x BRRI dhan28 (values with the same letter in a column are not significantly different at 5% level of significance).

Treatment	BC_1F_2 Genotypes/ Progenies	Plant Height (cm)	Productive Tillers (no/plant)	Panicle Length (cm)	Filled Spikelets (no/plant)	Unfilled Spikelets (no/plant)	Filled Spikelets (%)	Unfilled Spikelets (%)	Grain Yield (g/plant)	Na-K Ratio
Selected Genotypes										
No-Stress	Control	108.8^{a}	16.5^{a}	26.0^{a}	528.0^{a}	870.8^{a}	38.1^{a}	61.9^{b}	14.0^{a}	
Salinity-Stress	Tolerant	105.5^{a}	15.2^{b}	25.0^{b}	525.7^{a}	774.5^{b}	40.4^{a}	59.6^{b}	11.9^{b}	0.256^{a}
	Sensitive	84.9^{b}	9.5^{c}	21.9^{c}	78.7^{b}	507.2^{c}	12.7^{b}	87.3^{a}	1.5^{c}	0.311^{b}
$LSD_{0.05}$		4.41	1.09	0.76	61.79	90.60	7.05	7.05	1.35	0.05
All Genotypes										
No-Stress		108.8^{A}	16.5^{A}	26.0^{A}	528.0^{A}	870.8^{A}	38.1^{A}	61.9^{B}	14.0^{A}	
Salinity Stress		96.4^{B}	12.6^{B}	24.3^{B}	265.3^{B}	738.9^{B}	25.5^{B}	74.5^{A}	5.7^{B}	
$LSD_{0.05}$		2.98	0.77	0.53	35.90	63.79	2.57	2.57	0.96	

3.4.1.2. Productive Tillers

The productive tillers of the BC_1F_2 progenies of CSR28 x BRRI dhan28 varied from 5 to 29 under no stress condition and from 2 to 27 per plant under salinity stress (Table 3.4, Figs. 3.9 and 3.10). The number of productive tillers per plant varied significantly between the control and salinity treatments (Fig. 3.10, Table 3.5). Under salinity stress, the interquartile range of the productive tillers was 7 for the progenies of CSR28 x BRRI dhan28and for the control it was 6.5 (Fig. 3.10). The median value of the productive tillers for control and stress condition was 16 and 13 per plant, respectively.

The variability in productive tillers among the families from the cross, CSR28 x BRRI dhan28 is shown in Fig. 3.11. Overall higher productive tillers were observed under no-stress condition than salinity stress. Among the families, IR129282-19 produced the highest number of productive tillers per plant.

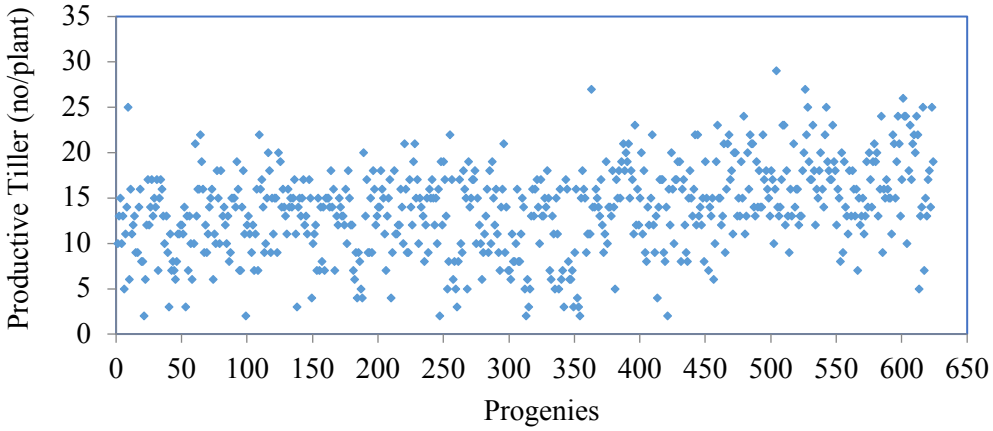
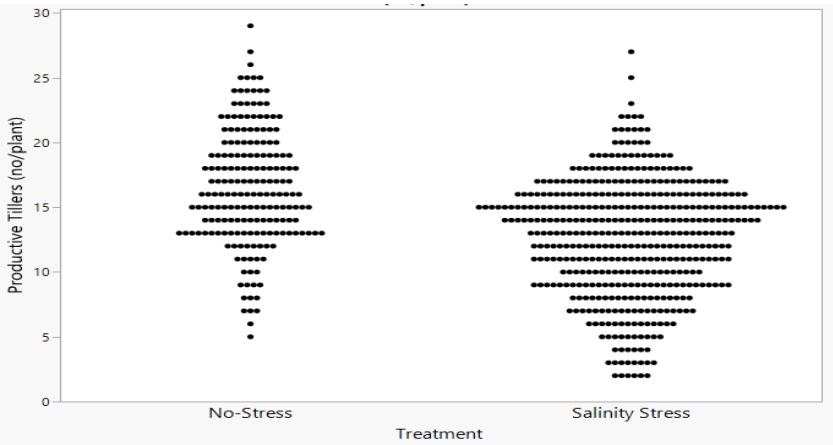


Figure 3.9. Number of productive tillers per plant of all the BC_1F_2 progenies of rice from the cross, CSR28 x BRRI dhan28.

a. Scatter plot



b. Box plot

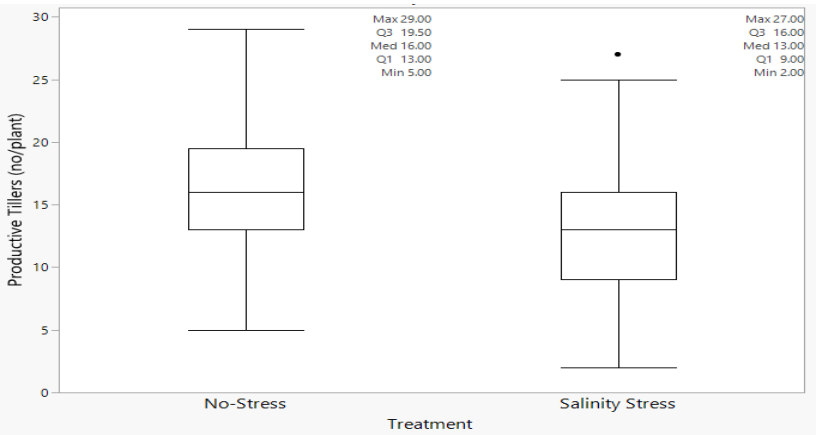


Figure 3.10. Scattered (a) and box (b) plots of the number of productive tillers per plant of BC_1F_2 individuals under control and salinity stress at reproductive stage of rice.

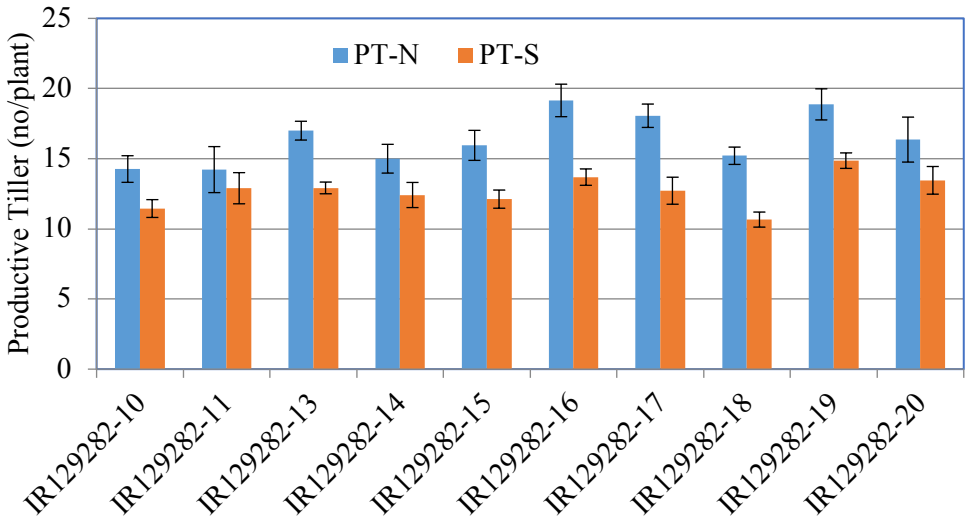


Figure 3.11. Mean productive tillers per plant of the rice families of CSR28 x BRRI dhan28 progenies under control (PT-N) and salinity stress (PT-S). Vertical and capped bar indicates standard error of the mean productive tillers of 7 to 85 plants per family.

The number of productive tillers of the tolerant and sensitive parents under control (no-stress) condition was 8 and 25 per plant, respectively (Table 3.4, Fig. 3.12). The mean productive tillers per plant of tolerant and sensitive progenies of CSR28 x BRRI dhan28 were 15.2 and 9.5, respectively and it was 16.5 tillers per plant under no-stress condition (Fig. 3.12). The reduction in productive tillers of tolerant and sensitive genotypes grown under salinity stress was about 14% and 36%, respectively over those grown under control condition (Table 3.4). Under salinity stress, the sensitive progenies produced 25.5% less productive tillers than the tolerant progenies of this cross. Under selective genotyping, the number of productive tillers per plant significantly differed between tolerant and sensitive individuals (Fig. 3.12; Table 3.5).

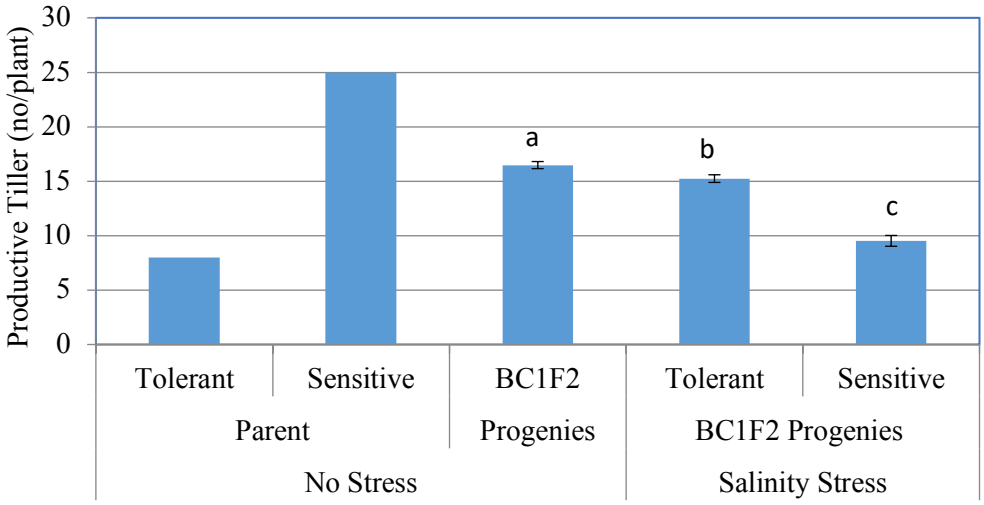


Figure 3.12. Mean productive tillers per plant of the tolerant and sensitive parents under control (no-stress) and their BC_1F_2 progenies under non-stress and salinity stress at reproductive stage of rice. Vertical and capped bar indicates standard error of the mean productive tillers of 189 plants grown under no-stress, 98 tolerant and 92 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

3.4.1.3. Panicle Length

The panicle length of the BC_1F_2 progenies of CSR28 x BRRI dhan28 varied from 12.3 cm to 34.9 cm under stress condition and it varied from 14.5 cm to 36.1 cm under no-stress condition (Table 3.4; Fig. 3.13). The mean panicle length of the progenies under no-stress condition was about 26 cm. Under salinity stress, the panicle length was about 25cm for tolerant and 21.9 cm for the sensitive progenies. Highly significant variation in panicle length was observed between no-stress and salinity treatments (Fig. 3.14; Table 3.5).

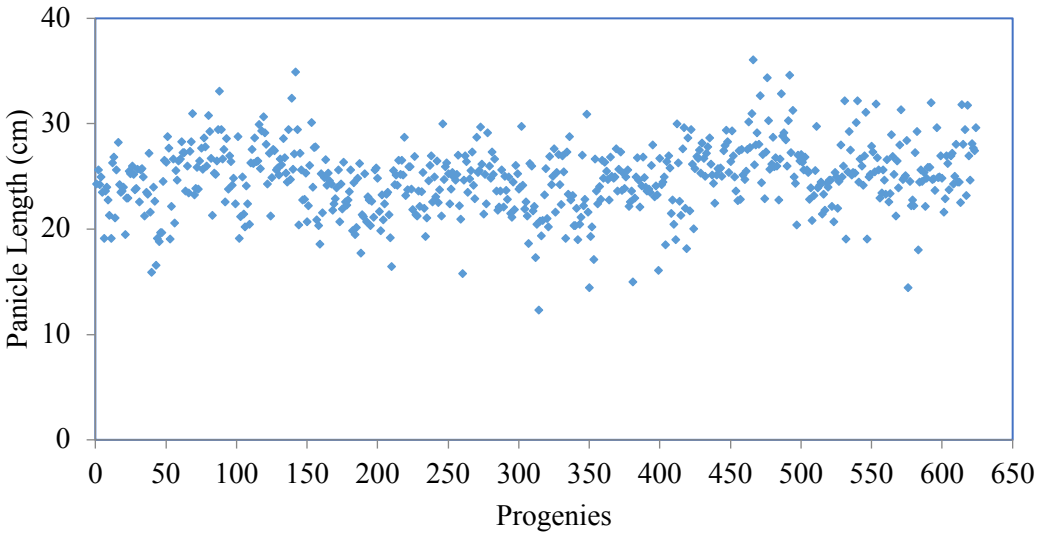
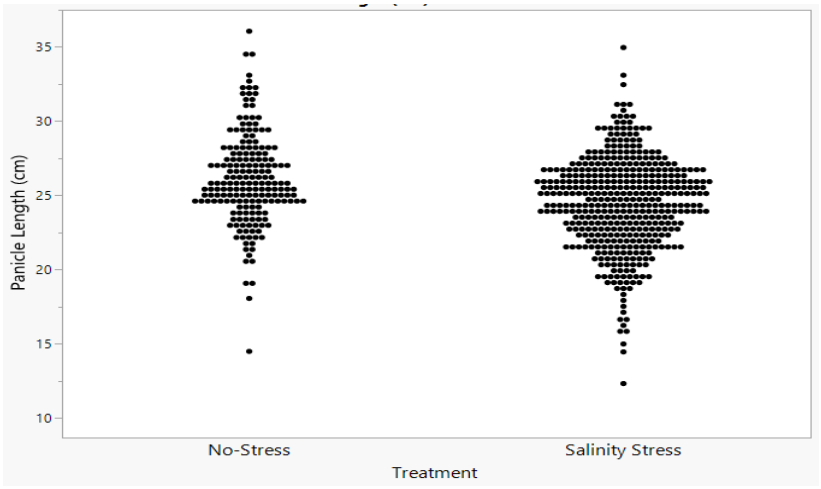


Figure 3.13. Panicle length of rice of all the BC_1F_2 individuals of CSR28 x BRRI dhan28.

The interquartile range for CSR28 x BRRI dhan28 progenies was 2.61 cm (no-stress) and 3.99cm (stress) (Fig. 3.14). The panicle length was variable among the families of the cross, CSR28 x BRRI dhan28 (Fig. 3.15). Overall, higher panicle length was observed among the plant families grown under no-stress condition than those grown under salinity stress. Among them, IR129282-13 and IR129282-20 produced the longest panicle and the shortest panicle was observed in IR129282-15.

a. Scatter plot



b. Box plot

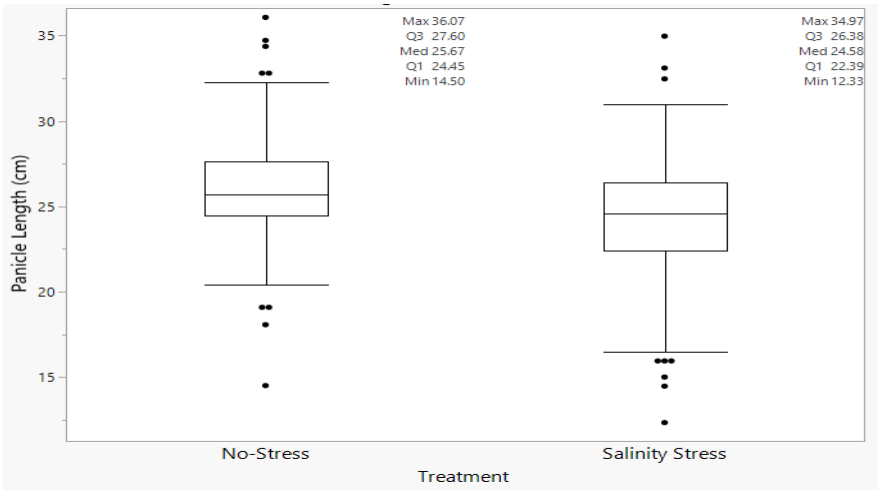


Figure 3.14. Scattered (a) and box (b) plots of the panicle length of BC_1F_2 individuals under normal and salinity stress at reproductive stage of rice.

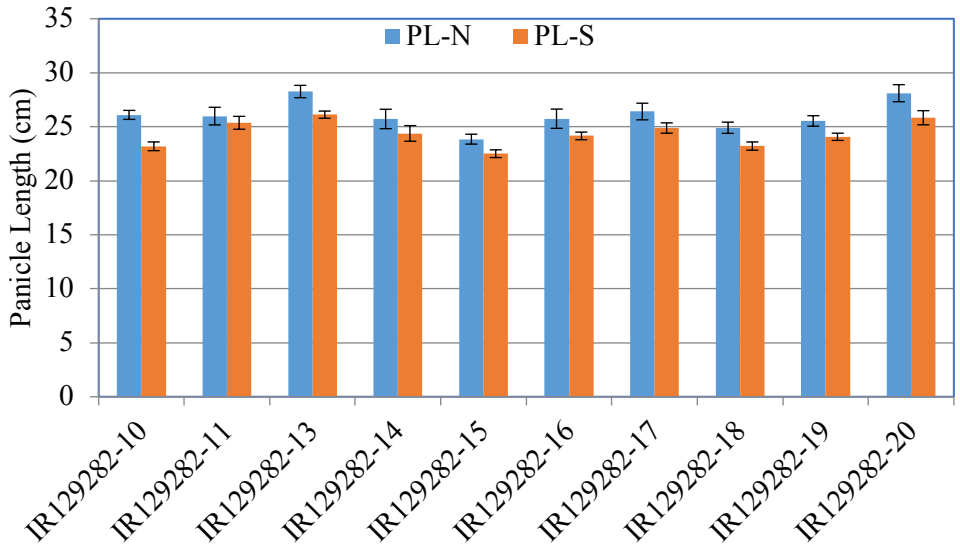


Figure 3.15. Mean panicle length of the rice families of CSR28 x BRRI dhan28 progenies under control (PL-N) and salinity stress (PL-S). Vertical and capped bar indicates standard error of the mean panicle length of 7 to 85 plants per family.

The panicle length of the tolerant and sensitive parents was 29 cm and 27 cm, respectively under control condition (Table 3.4, Fig. 3.16). Under selective genotyping, the panicle length of the CSR28 x BRRI dhan28 progenies varied significantly between tolerant and sensitive individuals and also between tolerant and control progenies (Fig. 3.16, Table 3.5). Under selective genotyping, the mean panicle length of tolerant and sensitive progenies for CSR28 x BRRI dhan28 under salinity stress was 25.0 cm and 21.9 cm, respectively; and it was 26.0 cm under no-stress condition. The reduction in panicle length of the tolerant and sensitive genotypes grown under salinity stress was about 4% and 16%, respectively over that grown under control condition (Fig. 3.16). Under salinity stress, the sensitive progenies had 12.6% shorter panicles than the tolerant progenies of this cross (Table 3.4).

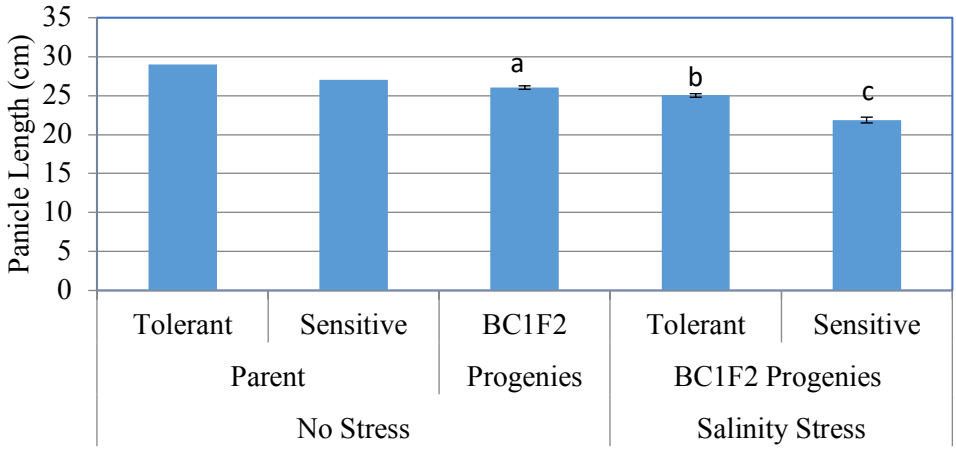


Figure 3.16. Mean panicle length of the tolerant and sensitive parents under no-stress condition and their BC_1F_2 progenies under no-stress and salinity stress at reproductive stage of rice. Vertical and capped bar indicates standard error of the mean panicle length of 189 plants grown under no-stress, 98 tolerant and 92 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

3.4.1.4. Number of Filled Spikelets

The number of filled spikelets of the BC_1F_2 progenies of CSR28 x BRRI dhan28 varied from7 to 1216 per plant under no-stress condition, while it varied from 0 to 1009 filled spikelets per plant under salinity stress (Table 3.4, Fig. 3.17). Highly significant ($p<0.001$) variation in the number of filled spikelets per plant was observed between control and salinity treatments (Fig. 3.18, Table 3.5).

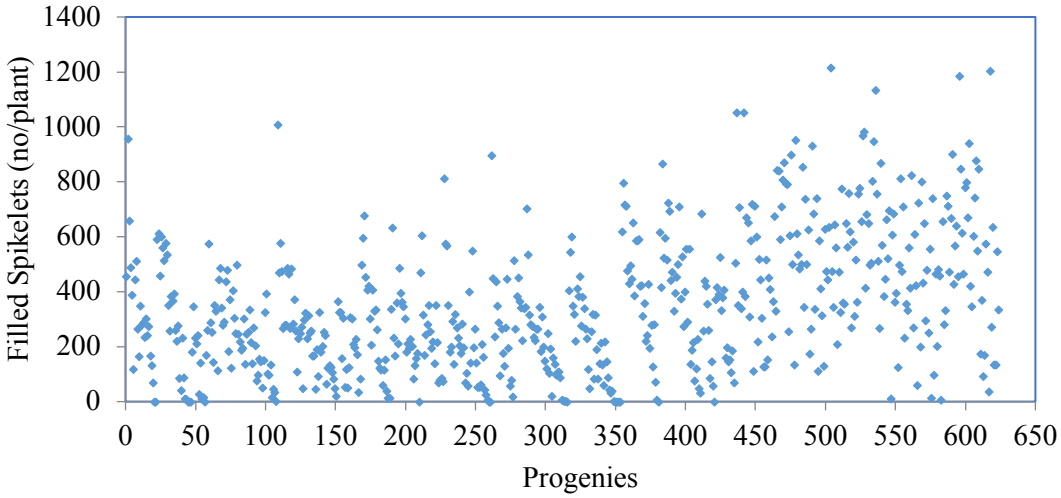
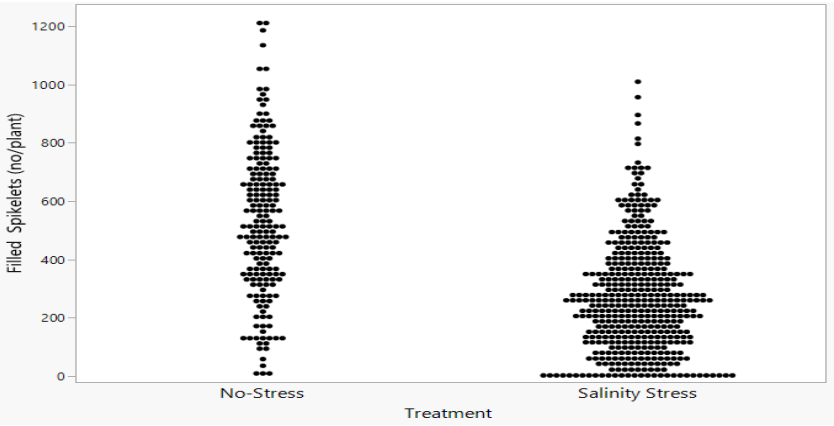


Figure 3.17. Number of filled spikelets per plant of all the BC_1F_2 individuals from the cross, CSR28 x BRRI dhan28.

The interquartile range of the filled spikelets per plant of the progenies CSR28 x BRRI dhan28 was 250 under salinity stress (Fig. 3.18) and under no stress condition, number of filled spikelets varied from 343-709 per plant indicated that interquartile range of control treatment was wider than the stress condition. The number of filled spikelets per plant varied significantly between no-stress and salinity treatments (Fig. 3.18, Table 3.5).

The number of filled spikelets per plant varied among the families of the cross, CSR28 x BRRI dhan28 (Fig. 3.19). The plant families produced more filled spikelets per plant under no-stress. Among the families, IR129282-16produced the highest and IR129282-11produced the lowest number of filled spikelets per plant, respectively.

a. Scatter plot



b. Box plot

Figure 3.18. Scattered (a) and box (b) plots of the number of filled spikelets per plant of BC_1F_2 individuals under normal and salinity stress at reproductive stage of rice.

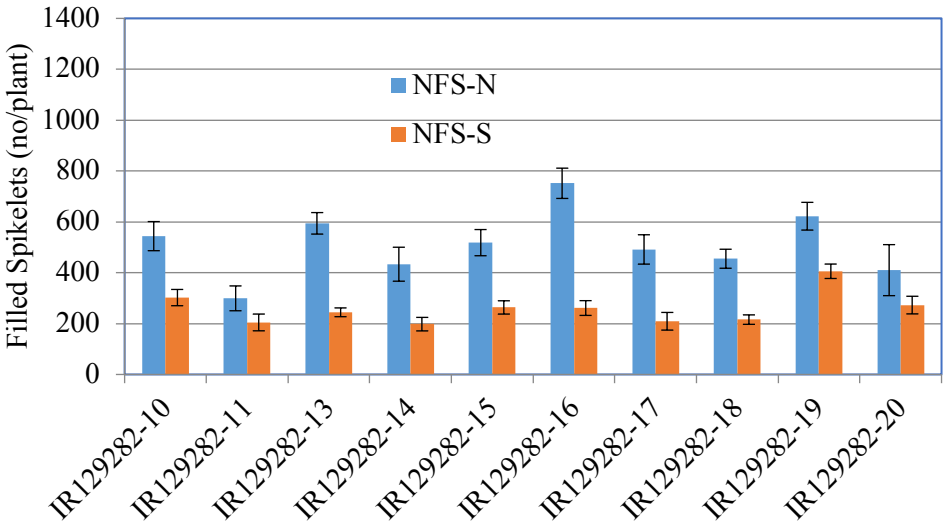


Figure 3.19. Mean number of filled spikelets per plant of rice families of CSR28 x BRRI dhan28 progenies under control (NFS-N) and salinity stress (NFS-S). Vertical and capped bar indicates standard error of the mean number of filled spikelets of 7 to 85 plants per family.

The number of filled spikelets of the tolerant and sensitive parents was 957 and 2349 per plant, respectively under no-stress condition (Table 3.4, Fig. 3.20). Under selective genotyping, the number of filled spikelets per plant of the tolerant and sensitive progenies of CSR28 x BRRI dhan28 was 525.7 and 78.7, respectively; and it was 528.0 per plant under no-stress condition (Fig. 3.20). The number of filled spikelets per plant varied significantly between tolerant and sensitive individuals, but no significant variation was observed between tolerant progenies and those grown under no-stress condition (Fig. 3.20, Table 3.5).

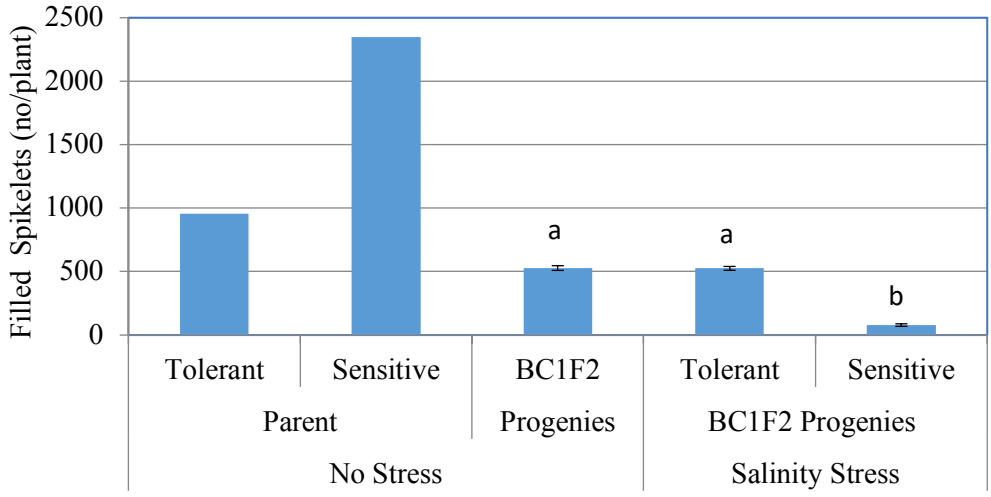


Figure 3.20. Number of filled spikelets per plant of tolerant and sensitive parents under no-stress and their BC_1F_2 progenies under no-stress and salt stress at reproductive stage of rice. Vertical and capped bar indicates standard error of the mean number of filled spikelets of 189 plants grown under no-stress, 98 tolerant and 92 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

3.4.1.5. Number of Unfilled Spikelets

The number of unfilled spikelets of the BC_1F_2 progenies of CSR28 x BRRI dhan28 varied from 103 to 2769 per plant under no-stress, and from 76 to 2114 per plant under salinity stress conditions (Fig. 3.21, Table 3.4). It varied significantly ($p<0.001$) between no-stress and salinity treatments (Fig. 3.22, Table 3.5).

The interquartile range of the unfilled spikelets per plant of the progenies CSR28 x BRRI dhan28 was 459 under salinity stress, but slightly higher values were observed under no-stress (544) treatment (Fig. 3.22). The median value was also slightly higher in control (831) condition over the salinity stress (703 unfilled spikelets per plant).

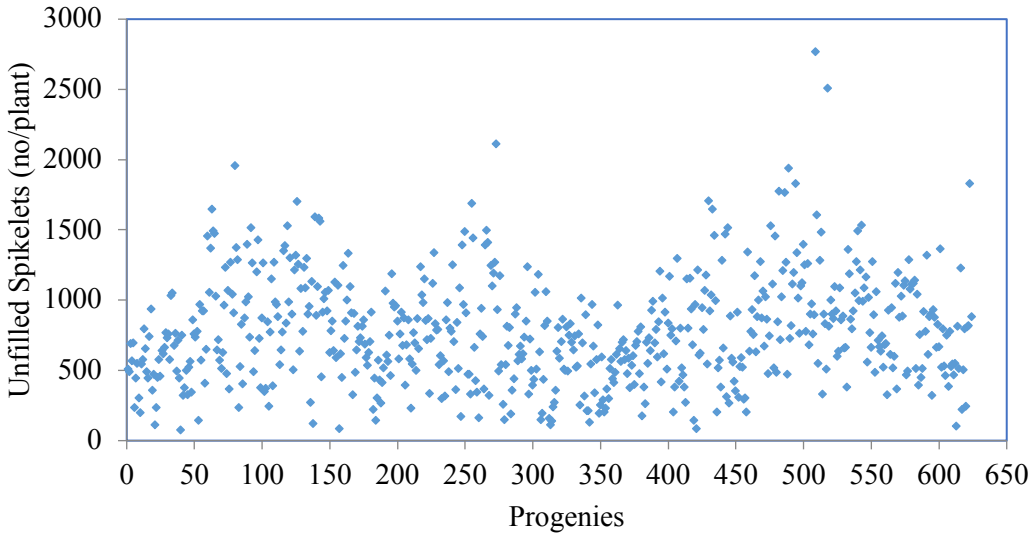
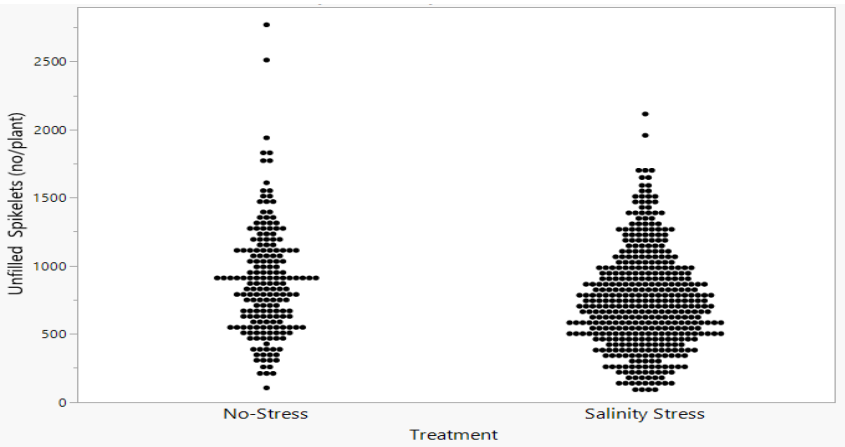


Figure 3.21. Number of unfilled spikelets per plant of all the BC_1F_2 individuals from the cross, CSR28 x BRRI dhan28 at reproductive stage of rice.

The variability in the number of unfilled spikelets per plant among the families from the crosses of CSR28 x BRRI dhan28 is shown in Fig. 3.23. No definite pattern followed in case of the number of filled spikelets between the control and salinity treatment. Among them, the highest and lowest number of unfilled spikelets was observed in the family, IR129282-14 and IR129282-10, respectively.

a. Scatter plot



b. Box plot

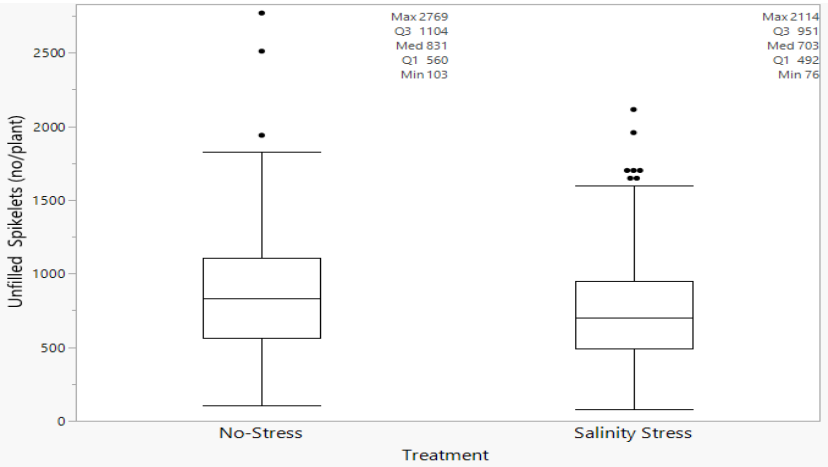


Figure 3.22. Scattered (a) and box (b) plots of the number of unfilled spikelets per plant of BC_1F_2 individuals under normal and salinity stress at reproductive stage of rice.

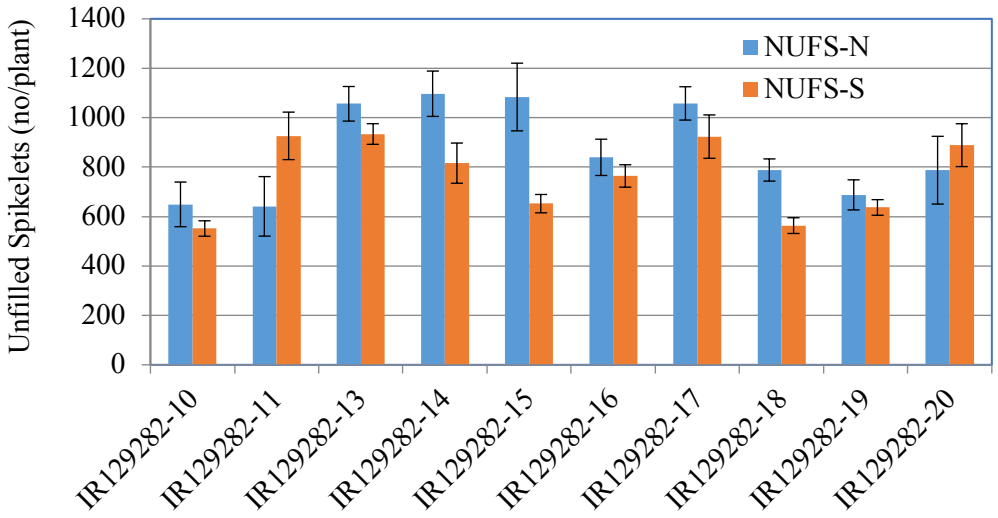


Figure 3.23. Mean number of unfilled spikelets per plant of rice families of CSR28 x BRRI dhan28 progenies under control (NUFS-N) and salinity stress (NUFS-S). Vertical and capped bar indicates standard error of the mean number of unfilled spikelets of 7 to 85 plants per family.

The number of unfilled spikelets of the tolerant and sensitive parents was 451 and 617 per plant, respectively under no-stress condition (Table 3.4, Fig. 3.24). Under selective genotyping, the mean unfilled spikelets per plant of the tolerant and sensitive progenies for CSR28 x BRRI dhan28 were 774.5 and 507.2, respectively and it was 870.8 per plant under no-stress condition (Fig. 3.24). The number of unfilled spikelets per plant varied significantly between the tolerant and sensitive individuals as well as between tolerant and control treatments (Fig. 3.24, Table 3.5).

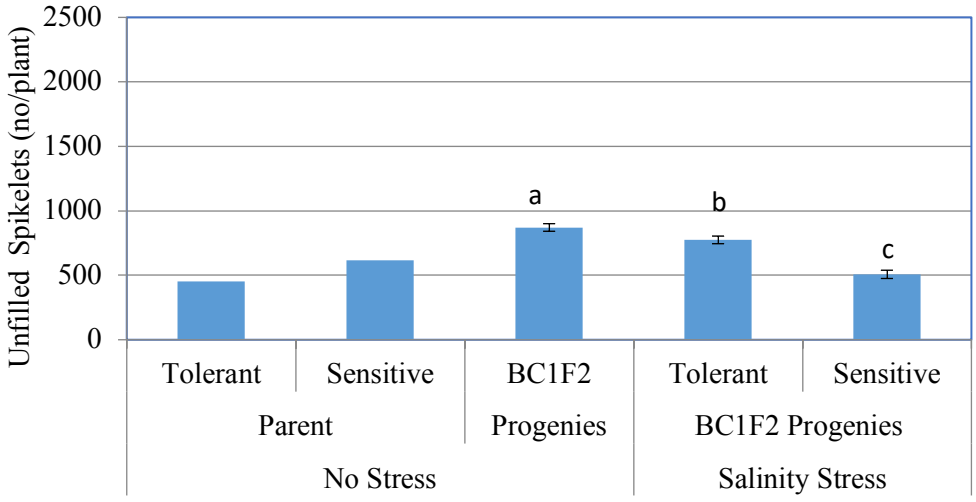


Figure 3.24. Number of unfilled spikelets per plant of the tolerant and sensitive parents under no-stress and their BC_1F_2 progenies under no-stress and salinity stress at the reproductive stage of rice. Vertical and capped bar indicates standard error of the mean number of unfilled spikelets of 189 plants grown under no-stress, 98 tolerant and 92 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

3.4.1.6. Percent Filled Spikelets

The percent filled spikelets of the BC_1F_2 progenies of CSR28 x BRRI dhan28 varied from 1.3% to 72.2% under no-stress condition, while it varied from 0to 72.7% under salinity stress (Fig. 3.25, Table 3.4). Significant difference was observed between no-stress and salinity stress treatment (Fig. 3.26, Table 3.5).

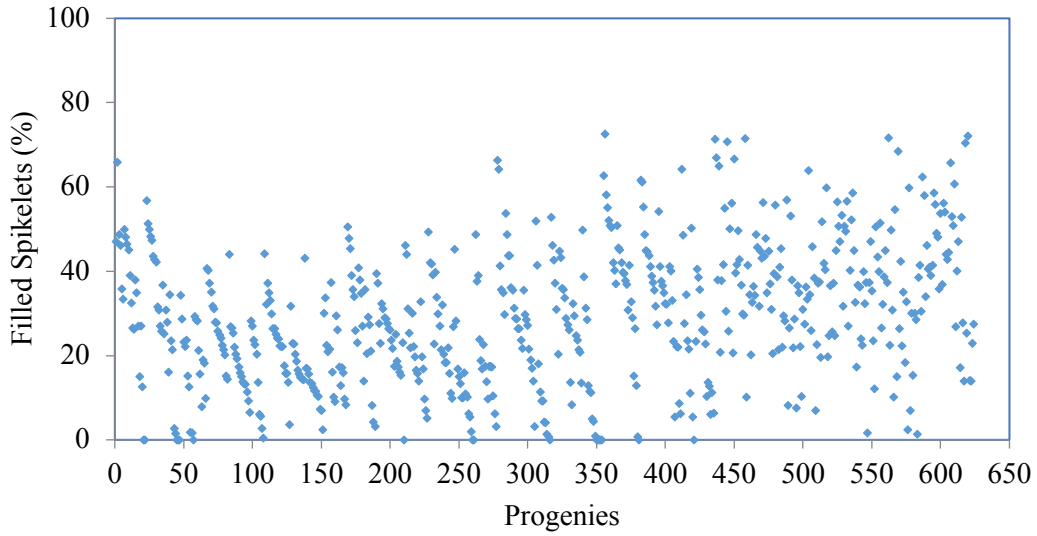
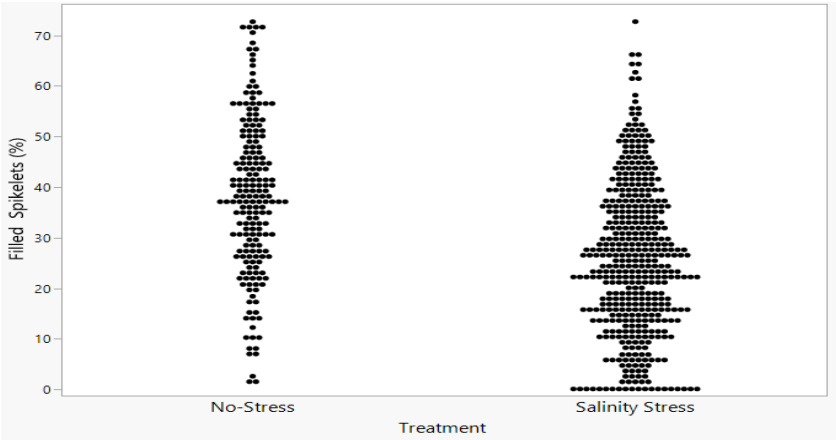


Figure 3.25. Percent filled spikelets per plant of all the BC_1F_2 individuals from the cross, CSR28 x BRRI dhan28.

Under salinity stress, the interquartile range of percent filled spikelets per plant of the progenies from CSR28 x BRRI dhan28 was 21.3% and under no-stress condition, it was 21.7% (Fig. 3.26). The median values of the progenies for the salinity and control treatment was 24.9 and 37.6, respectively. This implies that percent filled grains with salinity treatment were significantly reduced over the no-stress treatment (Table 3.5, Fig. 3.26).

The percent filled spikelets varied among the families of CSR28 x BRRI dhan28 (Fig. 3.27). Overall, percent filled spikelets were higher under no-stress than salinity stress. Among them, IR129282-16, 19 and 10 produced comparatively more filled spikelets than other plant families, while IR129282-17 produced the lowest percentages of filled spikelets under salinity stress.

a. Scatter plot



b. Box plot

Figure 3.26. Scattered (a) and box (b) plots of percent filled spikelets per plant of BC_1F_2 individuals under normal and salinity stress at reproductive stage of rice.

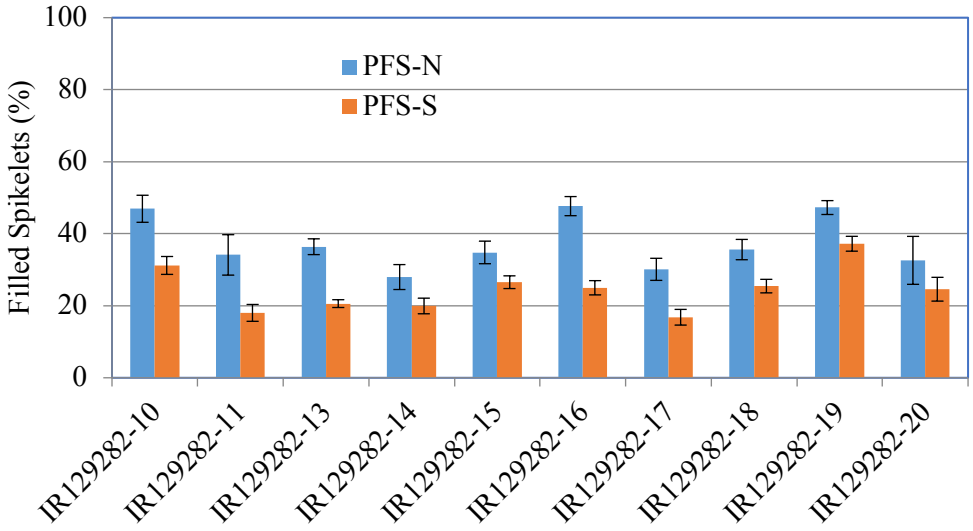


Figure 3.27. Mean percent filled spikelets of rice families of CSR28 x BRRI dhan28 progenies under control (PFS-N) and salinity stress (PFS-S). Vertical and capped bar indicates standard error of the mean percent filled spikelets of 7 to 85 plants per family.

The percent filled spikelets of the tolerant and sensitive parent was 68.0% and 79.2% under no-stress condition, respectively (Table 3.4; Fig. 3.28). Under selective genotyping, the mean of percent filled spikelets of the tolerant and sensitive progenies was 41.5% and 12.5%, respectively; and it was 38.1% under no-stress condition (Fig. 3.28). The percent filled spikeletes varied significantly between tolerant and sensitive, and no-stress and sensitive progenies of CSR28 x BRRIdhan28; but no significant variation observed between no-stress and tolerant progenies of CSR28 x BRRI dhan28 (Fig. 3.28, Table 3.5).

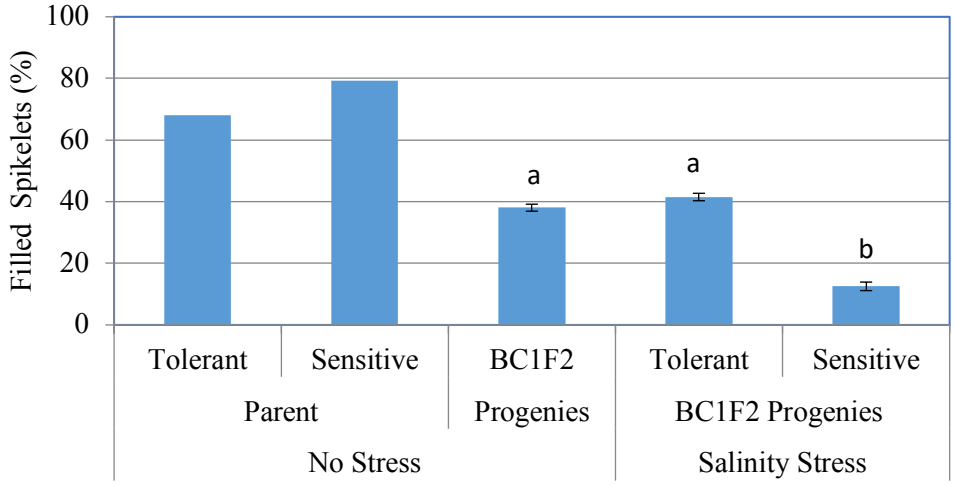


Figure 3.28. Percent filled spikelets per plant of tolerant and sensitive parents under no-stress and their BC_1F_2 progenies under no-stress and salt stress at the reproductive stage of rice. Vertical and capped bar indicates standard error of the mean percent filled spikelets of 189 plants grown under no-stress, 98 tolerant and 92 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

3.4.1.7. Grain Yield

The grain yield of BC_1F_2progenies from the cross, CSR28 x BRRI dhan28 is shown in (Figs. 3.29 and 3.30). The yield of the progenies varied from 2.3 to 36.9 g/plant under no stress and from 0.0 to 25.1 g/plants under salinity stress (Table 3.4, Fig. 3.30). Grain yield differed significantly ($p<0.01$) between control and salinity treatments (Fig. 3.30, Table 3.5). This indicates the existence of genetic variability among the progenies of CSR28 x BRRI dhan28.

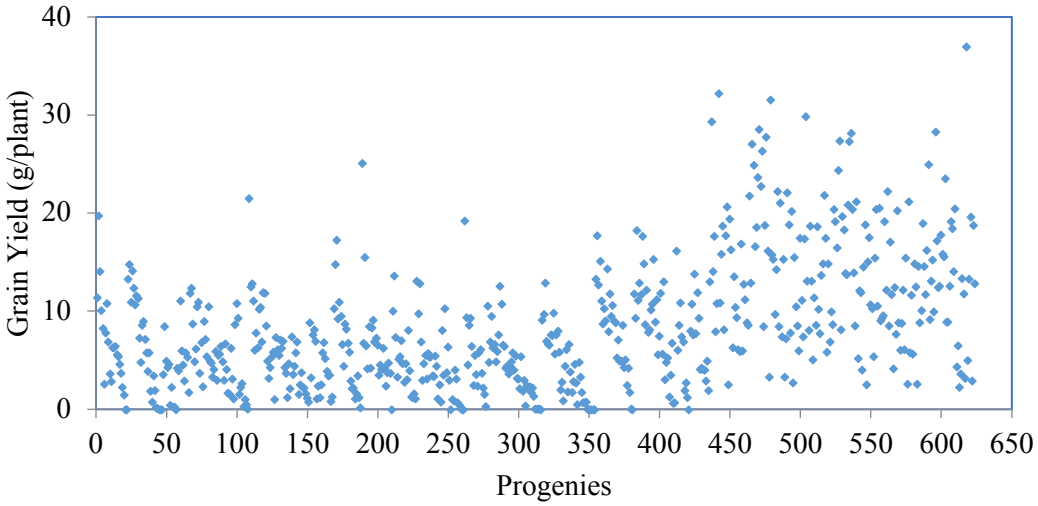
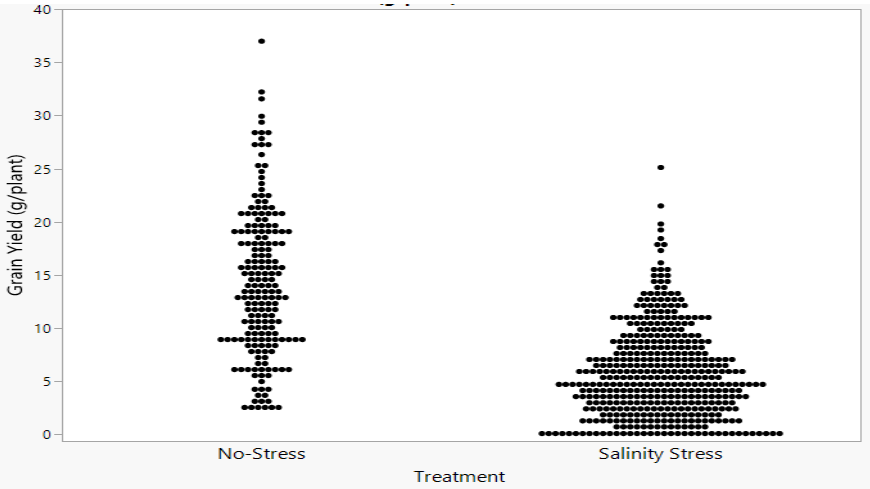


Figure 3.29. Grain yield per plant of all the BC_1F_2 individuals from the cross, CSR28 x BRRI dhan28.

The interquartile range of the grain yield for the stressed treatment was 5.67 g/plant and under no-stress condition; it was 10.1 g/plant (Fig. 3.30). The median of the progenies under stress and no-stress condition was 4.95 g/plant and 13.29 g/plant, respectively.

The grain yield varied among the families of the cross, CSR28 x BRRI dhan28 (Fig. 3.31). Overall, higher grain yield was observed among the plant families under control (no-stress) than the salinity treatment. The highest grain yield was obtained from the plant family, IR129282-16 under control and the lowest by IR129282-11 under salinity stress. This indicates genetic variability among the plant families of this cross.

a. Scatter plot



b. Box plot

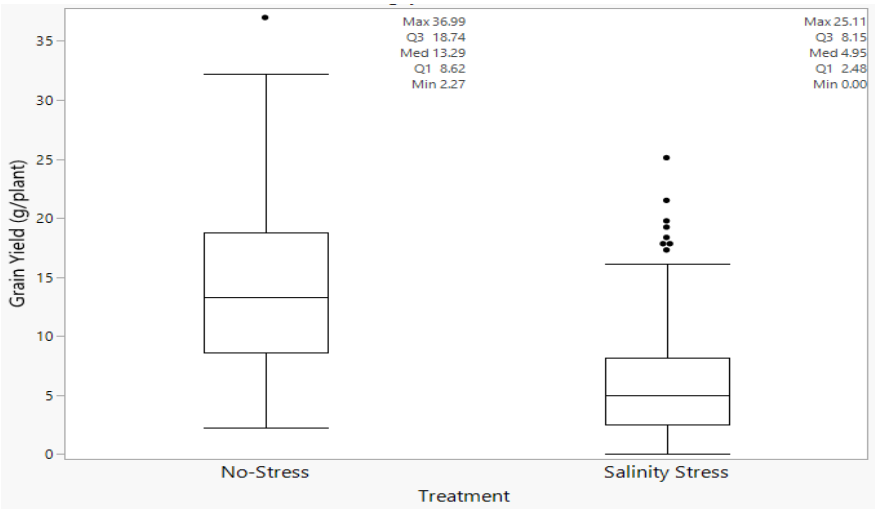


Figure 3.30. Scattered (a) and box (b) plots of the grain yield per plant of BC_1F_2 individuals under normal and salinity stress at reproductive stage of rice.

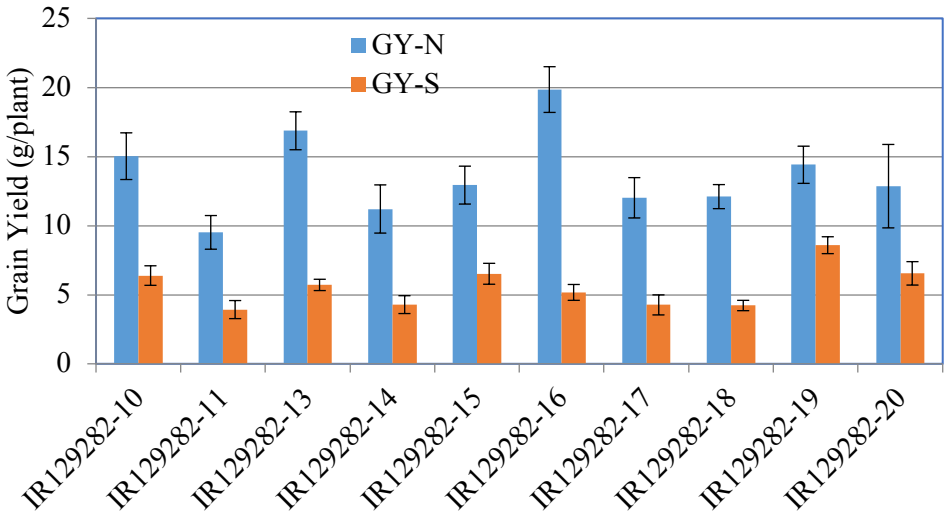


Figure 3.31. Mean grain yield of rice plant families of CSR28 x BRRI dhan28 progenies under control (GY-N) and salinity stress (GY-S). Vertical and capped bar indicates standard error of the mean grain yield of 7 to 85 plants per family.

The grain yield of tolerant and sensitive parents under normal growing condition (no-stress) was 17.5 g/plant and 55 g/plant, respectively (Fig. 3.32, Table 3.4). Under selective genotyping, mean grain yield of the tolerant and sensitive progenies of CSR28 x BRRI dhan28 was 11.9 g/plant and 1.5 g/plant, respectively (Fig. 3.32). And it was 14 g/plant under no-stress treatment, which was significantly different than that obtained under salinity stress (Table 3.5). The reduction in grain yield of the tolerant and sensitive progenies was 15.8% and 89.2%, respectively over the progenies grown under control condition (Table 3.4, Fig. 3.32).

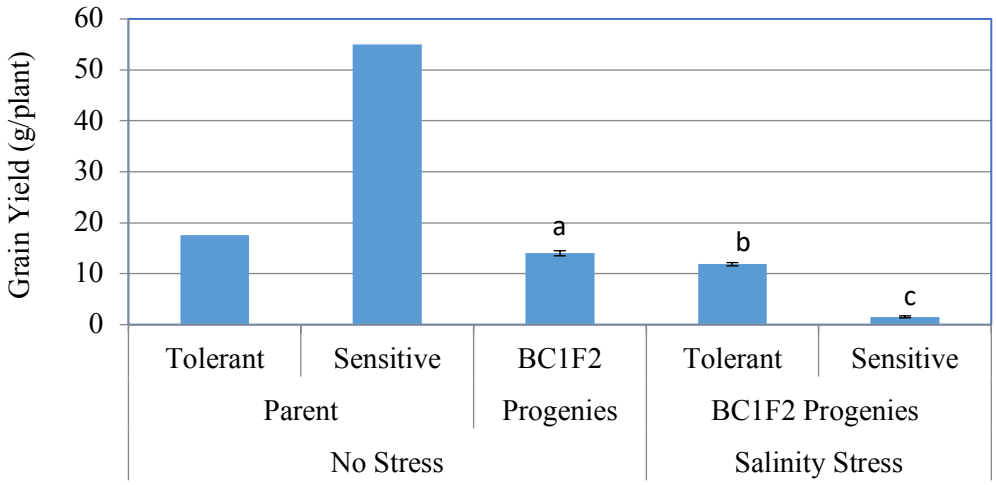


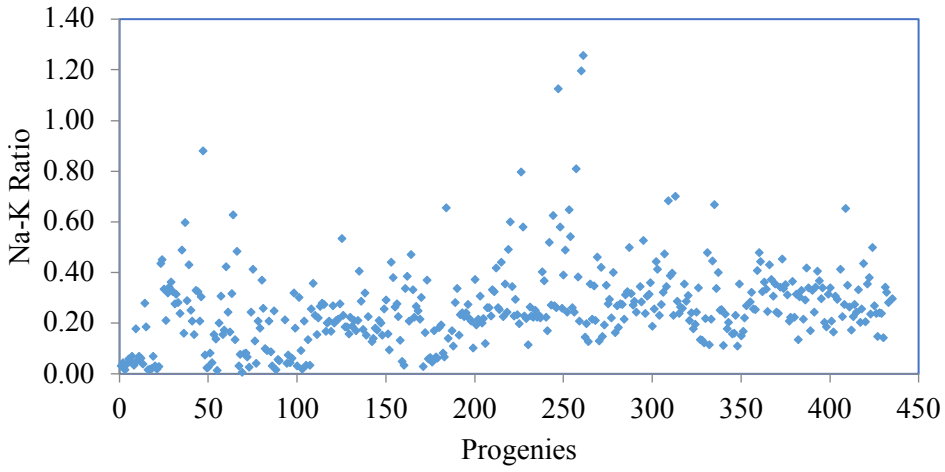
Figure 3.32. Grain yield of tolerant and sensitive parents under no-stress and their BC_1F_2 progenies under no-stress and salt stress condition at the reproductive stage of rice. Vertical and capped bar indicates standard error of the mean grain yield of 189 plants grown under no-stress, 98 tolerant and 92 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

3.4.1.8. Sodium-Potassium (Na^+-K^+) Ratio

The Na^+ concentration in the flag leaves of the BC_1F_2 progenies of CSR28 x BRRI dhan28 varied from 0.004 to 0.988 mmol/g and K^+ concentration varied from 0.035 to 3.22 mmol/g (Fig. 3.33). In tolerant progenies, the concentration of K^+ was higher than that of Na^+.

The lowest Na^+-K^+ ratio was recorded in the plant family, IR129282-13 and the highest in IR129282-17 under salinity stress (Fig. 3.34). The Na^+-K^+ ratio of the BC_1F_2 progenies of CSR28 x BRRI dhan28 varied from 0.005 to 1.26 under salinity stress of EC 10 dS/m at the reproductive stage. The Na^+-K^+ concentration of the tolerant and sensitive progenies was 0.26 and 0.31, respectively (Fig. 3.35).

a. Scatter plot



b. Box Plot

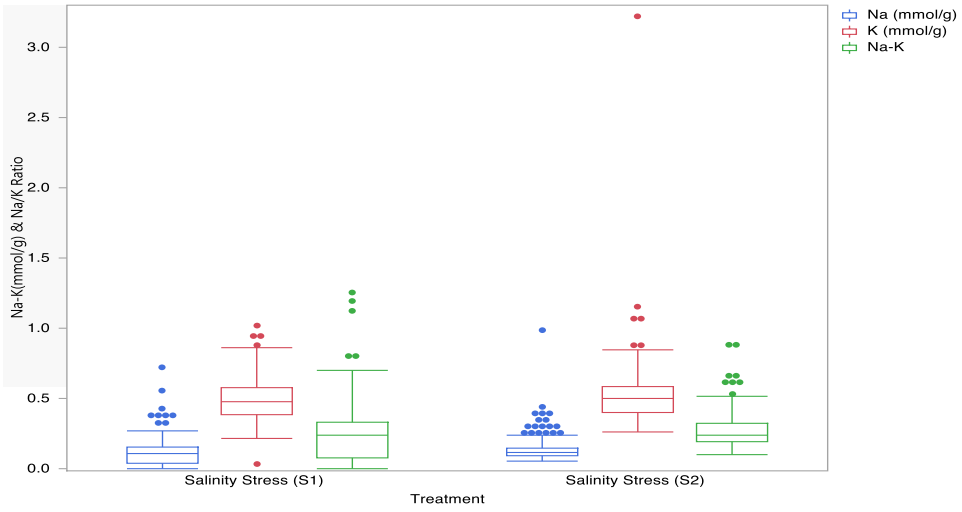


Figure 3.33. Scattered (a) and box (b) plots of Na-K ratio of the salinity-stressed BC_1F_2 progenies from the cross, CSR28 x BRRI dhan28.

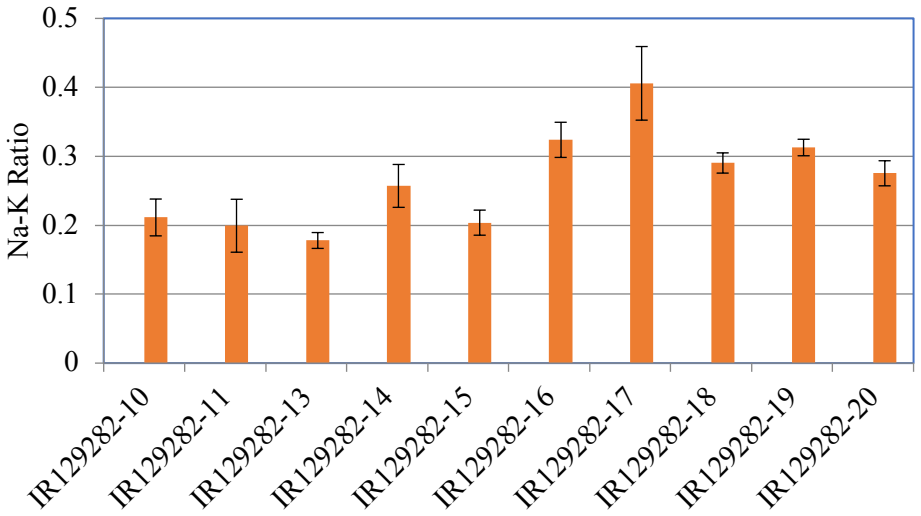


Figure 3.34. Mean Na-K ratio of rice families of CSR28 x BRRI dhan28 progenies under salinity stress. Vertical and capped bar indicates standard error of the mean Na-K ratio of 7 to 82 plants per family.

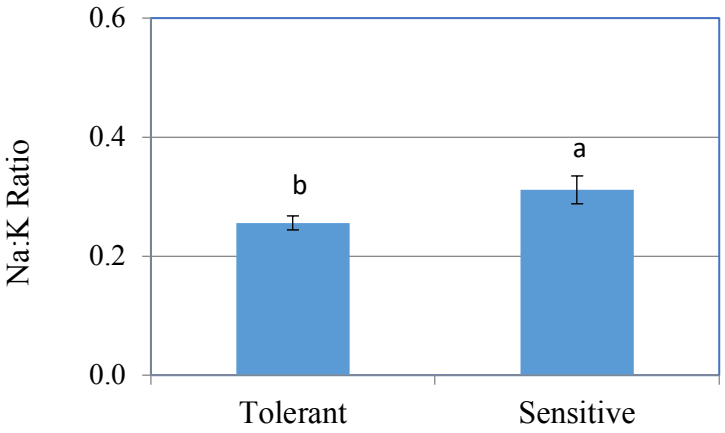


Figure 3.35. Mean Na-K ratio of the tolerant and sensitive BC_1F_2 progenies of the cross, CSR28 x BRRI dhan28. Vertical and capped bar indicates standard error of the mean Na-K ratio of 98 tolerant and 92 sensitive plants. Values with the same letter are not significantly different at 5% level of significance.

3.4.2. Correlation Analysis of Yield and Agronomic Components

Correlation coefficient analysis measures the association between two traits. When establishing the relationship between salt-stress and overall phenotypic performance, correlation analysis is useful (Gomez and Gomez, 1984; Sokal and Rohlf, 1995; Steel *et al.* 1997). It provides an indication of the degree of association between two variables that are considered to be independent. However, association detected from correlation coefficients may not necessarily be attributed to a single variable but rather to the number of interdependent variables.

Correlation analysis of the BC_1F_2 individuals of CSR28 x BRRI dhan28 was performed to determine relationship between grain yield with other yield contributing and agronomic components under salt-stress (Fig. 3.36). Highly significant positive correlations were observed between the grain yield and number of filled spikelets (r=0.93), percent filled spikelet (r=0.72), and number of productive tillers per plant (r=0.52); while the percent unfilled spikelets (r = -0.72) and SES score (r = -0.73) showed significantly negative correlation with the grain yield under salinity stress.

The number of unfilled spikelets per plant showed positive but weak correlation with grain yield, which is unexpected (Fig. 3.36). Mondal et al. (2019) observed similar positive correlation with grain yield and number of unfilled spikelets of the F_2 mapping population from the cross between NSIC Rc222 (sensitive parent) and BRRI dhan47 (tolerant parent). The possible reason may be the tendency of producing excess sterile spikelets by the tolerant parents under salt stressed environment.

Salinity evaluation score (SES) showed negative but highly significant correlation with the plant height, productive tiller per plant, panicle length, number of filled spikelets per plant, percent filled spikelets and grain yield (Fig. 3.36). And it showed highly significant positive correlation with percent unfilled spikelets. Therefore, SES score might be an initial stress indicator to identify salt-tolerant and salt-sensitive genotypes. In addition to SES score, grain yield, filled spikelets and productive tillers are considered the ultimate stress indicators for salinity tolerance in rice.

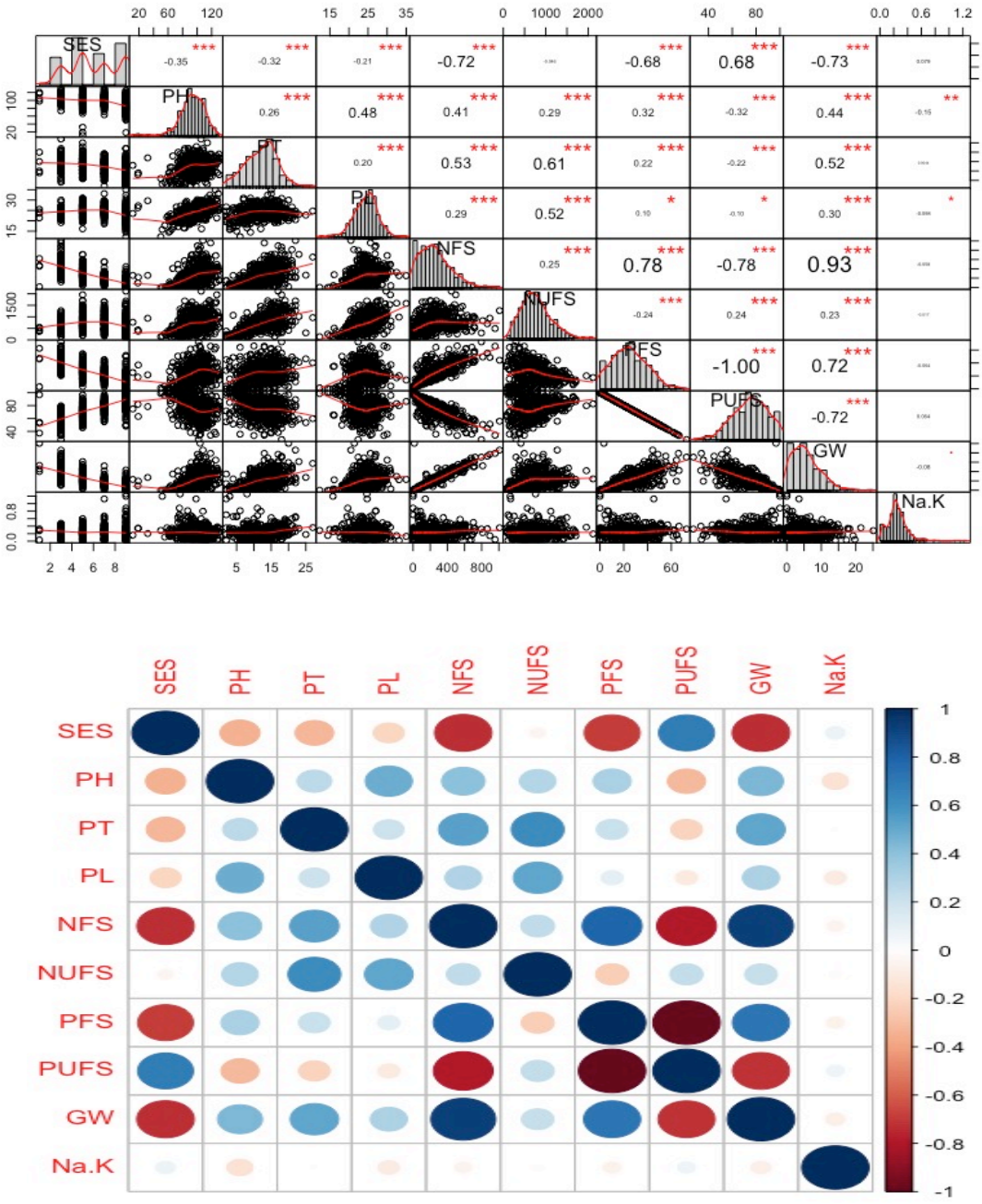


Figure 3.36. Phenotypic distribution and correlation coefficients for grain yield, agronomic and physiological components of BC_1F_2 individuals from CSR28 x BRRI dhan28. Pearson correlation coefficient (top) and correlogram (bottom) among the traits under salinity stress of 10 dS/m at the reproductive stage of rice plant.

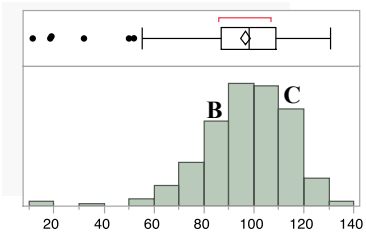
3.4.3. Frequency Distribution

In most cases, significant variation was observed for all traits in the BC_1F_2 population of CSR28 x BRRI dhan28 (Figs. 3.5-3.35). The variation of the BC_1F_2 population was beyond the range of their parents, suggesting transgressive segregation for a particular trait.

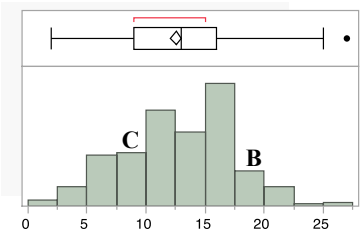
Out of the 435 BC_1F_2 progenies of CSR28 x BRRI dhan28, 98 highly tolerant and 92 most sensitive plants were selected based on selective genotyping. They represent two extreme tails of the phenotypic distribution, based on their responses to salt stress for the traits described in the subsequent sections.

3.4.3.1. Plant Height

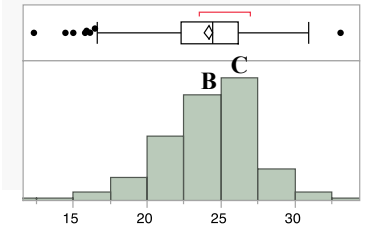
The segregation pattern for the plant height of BC_1F_2 population of CSR28 x BRRI dhan28 is shown in Fig. 3.37 Some BC_1F_2 progenies had higher plant height than the tolerant parent and some had lower than the sensitive parent under salinity stress. The plant height of the tolerant (CSR28) and sensitive (BRRI dhan28) parents were 112.8 cm and 73.0 cm, respectively (Fig. 3.37). The mean plant height the progenies was 96.4 cm that varied from 11.5 cm to 130.5 cm, with a median of 97.0 cm. The plant height followed negatively skewed distribution (-0.97) (Table 3.4). Most of the plant height (89%) ranged from 70 cm to 120 cm. Out of 435 BC_1F_2 progenies, the plant height of 70 individuals (16.1%) were taller than the tolerant parent, CSR28 and only 27 individuals (6.2%) had shorter plants than the sensitive parent, BRRI dhan28. Overall, 77.7% (338 individuals) of the total population had plant height in between the tolerant and sensitive plants.



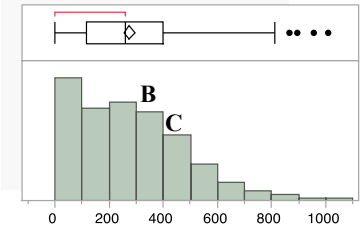
a. Plant Height (cm)



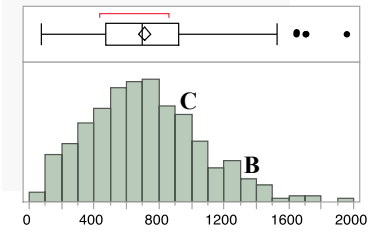
b. Productive Tiller (no/plant)



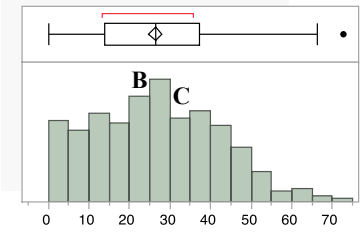
c. Panicle length (cm)



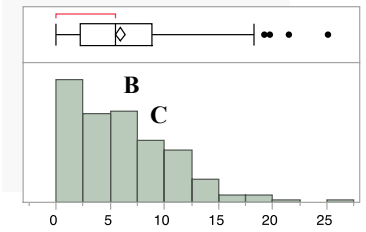
d. Filled Spikelets (no/plant)



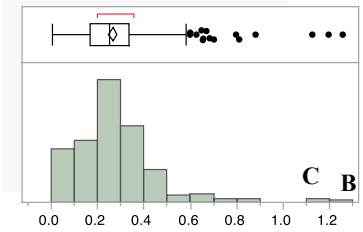
e. Unfilled Spikelets (no/plant)



f. Filled Spikelets (%)



g. Grain Yield (g/plant)



h. Na-K Ratio

Figure 3.37. Frequency distribution of the BC_1F_2 progenies of CSR28 x BRRI dhan28 under salinity stress of EC 10 dS/m at reproductive stage of rice plant.

3.4.3.2. Productive Tillers

The frequency distribution of number of productive tillers per hill of the BC_1F_2 population from the cross, CSR28 x BRRI dhan28 is shown in Fig 3.37. Some BC_1F_2 individuals had higher productive tillers than the tolerant parent and some had lower than the sensitive parent under saline stress.

The number of productive tillers of the tolerant and sensitive parents was 8 and 19 per plant, respectively (Fig. 3.37). The number of productive tillers per plant of the BC_1F_2 population of CSR28 x BRRI dhan28 ranged from 2 to 27 which follow the negatively skewed (-0.183) distribution (Table 3.4). Out of 435 BC_1F_2 progenies, 352 individuals (80.9%) had more productive tillers than the tolerant parent, while only 19 individuals (4.4%) had more productive tillers than the tolerant and sensitive parent (Fig. 3.37). Overall, 80.9% of the total population had productive tillers in between tolerant and sensitive parents (8-19 productive tillers/plant).

3.4.3.3. Panicle Length

The panicle length of the tolerant (CSR28) and sensitive (BRRI dhan28) parents was 26.2 cm and 24.5 cm, respectively (Fig. 3.37). The mean panicle length of the whole population was 24.3 cm and that ranged from 12.3 to 35.0 cm (Table 3.4). The panicle length of the first (Q1) and the third (Q3) quartiles was 22.4 cm and 26.4 cm, respectively under salinity stress (Fig. 3.14). Out of 435 BC_1F_2 progenies, 390 (89.7%) individuals had the panicle length ranging from 20 to 30 cm. Among them, 116 BC_1F_2 individuals had more panicle length than the tolerant (26.2 cm) and 213 individuals had less panicle length than the sensitive (24.5 cm) parent. This means 26.7% of the population had higher panicle length

than the tolerant parent, and 49.0% of the progenies had lower panicle length than sensitive parent. The panicle length of the BC_1F_2 population followed the negatively skewed (-0.30) distribution (Table 3.4). Overall, 105 individuals (24.1%) of the total population had panicle length within the range of the panicles produced by tolerant and sensitive parents.

3.4.3.4. Number of Filled Spikelets

The number of filled spikelets of the tolerant (CSR28) and sensitive (BRRI dhan28) parents was 451 and 356 per plant, respectively (Fig. 3.37). The mean of the whole population for this trait was 265.3 and that varies from 0.0 to 1009 spikelets per plant (Table 3.4). The number of filled spikeletsof the first and third quartile varied from 343 to 709 per plant under control, and it varies from 123 to 373 per plant under salinity stress (Fig 3.18) and the distribution is positively skewed (+0.83) (Table 3.4). Out of 435 BC_1F_2 progenies, 412 (94.7%) individuals had 0 to 600 filled spikelets per plant. Among them, 69 (15.9%) individuals had a greater number of filled spikelets per plant than the tolerant parent (451), while 317 individuals (72.9%) had a smaller number of filled spikelets per plant than the sensitive parent (356). Overall, only 11.3% (49 individuals) of the total population had the number of filled spikelets in between the tolerant and sensitive parents.

3.4.3.5. Number of Unfilled Spikelets

The number of unfilled spikelets of the tolerant (CSR28) and sensitive (BRRI dhan28) parents was 957 and 1377 per plant, respectively (Fig. 3.37). The mean of the whole population for this trait was 738.9 and that varied from 76 to 2114 unfilled spikelets per plant (Table 3.4). The number of unfilled spikelets of the first and third quartile varied from 560 to 1104 per plant under control, and it varies from 492 to 951 per plant under salinity stress with a median value of 831 and 703 unfilled spikelets per plant (Fig 3.22). The distribution is positively skewed (+0.60). Out of 435 BC_1F_2 progenies, 261 (69.9%) individuals had 0 to 1250 unfilled spikelets per plant. Overall, only 19.1% (83 individuals) of the total population had the number of unfilled spikelets in between the tolerant and sensitive parents. Among the BC_1F_2individuals, 327 (75.2%) individuals had a lesser number of unfilled spikelets per plant than the tolerant parent (957), CSR28 and only 25 individuals (5.7%) had a higher number of unfilled spikelets per plant than the sensitive parent (1377), BRRI dhan28. This implies that the progenies of CSR28 x BRRI dhan28 seem promising for development of salinity tolerance varieties.

3.4.3.6. Percent Filled Spikelets

The percentage of filled spikelets of the tolerant (CSR28) and sensitive (BRRI dhan28) parents was 32.03 and 20.56, respectively (Fig. 3.37). The mean of the whole population for this trait was 25.5 and that varies from 0 to 72.7% spikelets per plant (Table 3.4). The percent filled spikelets of the first and third quartile varied from 21.3% to 48.9% under control, and from 14.4% to 35.7% under salinity stress with a median value of 37.6% and 24.9%, respectively (Fig 3.26). The distribution of this population is positively skewed

(+0.33) (Table 3.4). Out of 435 BC_1F_2progenies, 417 (95.9%) individuals had percent filled spikelets ranged from 0 to 50%. Among them, 139 (32.0%) individuals had higher percentage of filled spikelets than the tolerant parent (32.03%), and 167 individuals (38.4%) had lower percentage of filled spikelets than the sensitive parent (20.56%). Overall, 29.7% (129 BC_1F_2 individuals) of the individuals had the filled spikelets in between tolerant and sensitive parents.

3.4.3.7. Grain Yield

The grain yield of the tolerant (CSR28) and sensitive (BRRI dhan28) parents was 9.95 g/plant and 6.45 g/plant, respectively (Fig. 3.37). The mean of the whole population for grain yield was 5.74 and that varied from 0 to 25.1 g/plant (Table 3.4). The grain yield of the first and third quartile varied from 8.6 g/plant to 18.7 g/plant under control, and it ranged from 2.5 g/plant to 8.2 g/plant under salinity stress with a median value of 13.3 g/plant and 4.9 g/plant, respectively (Fig 3.30). The distribution of grain yield this population is positively skewed (+0.96) (Table 3.4). Out of 435 BC_1F_2 progenies, 405 (93.1%) individuals had grain yield ranged from 0 to 12.5 g/plant. Among them, 72 (16.6%) BC_1F_2 individuals had higher grain yield per plant than the tolerant parent and 271 individuals (62.3%) had a lower grain yield per plant than the sensitive parent (Fig. 3.37). Overall, 21.1% (92 individuals) of the individuals produced grain yield in between tolerant and sensitive parents.

3.4.3.8. Sodium-Potassium (Na^+-K^+) Ratio

The Na^+-K^+ ratio of the tolerant (CSR28) and sensitive (BRRI dhan28) parents was 1.613 and 3.105 respectively (Fig. 3.37). The Na^+-K^+ ratio of CSR28 x BRRI dhan28 varied from 0.005 to 1.256. The distribution of this population is positively skewed (+1.902) (Table 3.4). Out of 435 BC_1F_2 progenies, the Na^+-K^+ concentration of 371 (85.3%) individuals ranged from 0 to 0.4.

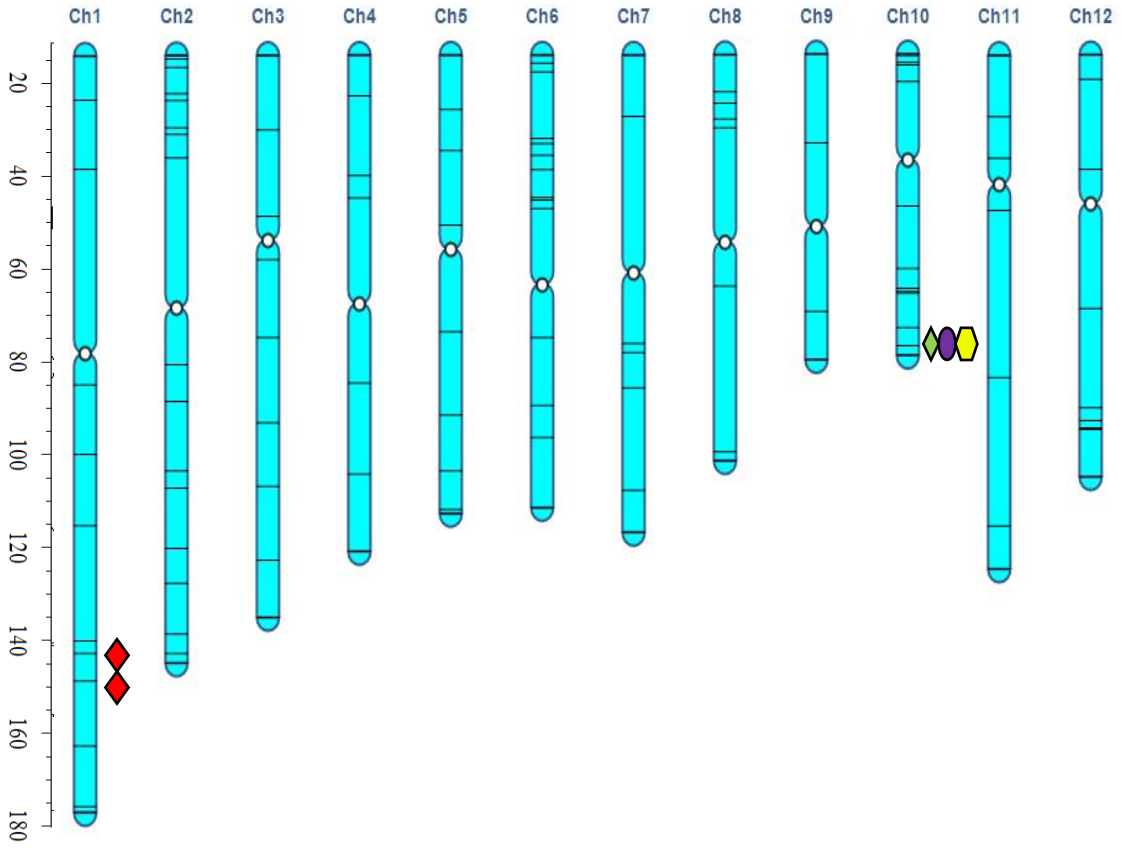
3.4.4. QTL Mapping for Salinity Tolerance at Reproductive Stage

QTLs related with yield and some important agronomic components of the BC_1F_2 progenies of CSR28 x BRRI dhan28 under salinity stress of 10 dS/m were identified through Inclusive composite interval mapping (ICIM) using IciMapping. In total, 15 QTLs were identified in seven traits: plant height (PH), panicle length (PL), number of filled spikelets (NFS), number of unfilled spikelets (NUFS), percent filled spikelet (PFS), grain yield (GY) and sodium-potassium (Na^+-K^+) ratio. The QTLs were detected on six chromosomes only (Table 3.6). The phenotypic variations of the identified QTLs individually accounted for 0.49% to 14.49%. Table 3.6 represents the name of the QTLs identified, chromosomal location, nearest marker interval, peak LOD, phenotypic variation explained by QTL (R^2) and direction of the phenotypic effect (additive effect) and allelic effect.

3.4.4.1. Linkage Analysis

Linkage maps of 12 chromosomes were created based on Kosambi functions and genotypic data of 190 BC_1F_2 individuals from cross, CSR28 x BRRI dhan28, with 116 polymorphic SNP markers using IciMapping software (Fig. 3.38). A LOD value of 3.0 was

used for estimation of map distance.QTL mapping was done with IciMapping (ICIM) software and ICIM-ADD methods were used for identifying putative QTLs. The percentage of total phenotypic variation explained by QTL identified for each trait was estimated as R^2 value. The data were permuted 1000 times to confirm the presence of each QTL across the 12 chromosomes. Total length of the distribution of the 116 polymorphic markers was 357.17 Mb or 1428.69 cM.



Symbol	Traits
◆	Plant Height
◆	No Filled Spikelets
●	% Filled Spikelets
⬢	Grain Yield

Figure 3.38. Genetic linkage map of 12 chromosomes based on the BC_1F_2 mapping population of CSR28 x BRRI dhan28 under salinity stress of EC 10 dS/m at reproductive stage of rice. Significant QTLs are shown at the right side of each chromosome based on the physical position (cM) of the SNP markers. Ch represents chromosomes and cM represents position in centimorgan.

3.4.4.2. Plant Height

For plant height, two significant QTLs ($p<0.05$) were identified on the chromosome 1 under salinity stress at the reproductive stage of rice (Table 3.6, Figs. 3.38 and3.39). The QTLs, *qPH1.1* and *qPH1*.2 were mapped on the long arm of the chromosome 1 at 142.40 cM, and 148.40 cM, respectively having similar LOD value (~5.70). But the QTL, *qPH1.1* can explain 5.75% and *qPH1.2* can explain 8.92% phenotypic variation having opposite additive effect indicated that both tolerant (CSR28) and sensitive (BRRI dhan28) parents were responsible for the allele contribution (Table 3.6).

3.4.4.3. Panicle Length

Only one QTL was identified for panicle length on the short arm of chromosome 3 with a LOD value of 3.02 that can explain 7.13% phenotypic variation (Table 3.6, Figs. 3.38 and 3.40). This QTL exhibited negative additive effect indicated that the tolerant parent, CSR28 was responsible for the allele contribution.

Table 3.6. QTLs identified using Inclusive Composite Interval Mapping (IciMapping) for agronomic components of the BC_1F_2 progenies from CSR28 x BRRI dhan28 under salt stress of EC 10 dS/m at the reproductive stage of rice.

Trait Name	Chromo-some	QTL	Position (cM)	Left Marker	Right Marker	LOD	PVE (%)	Additive Effect	Allelic Effect
Plant Height	1	*qPH1.1*	142.40	K_id1020667	K_id1021040	5.71	5.75	-10.90	CSR28
	1	*qPH1.2*	148.40	K_id1021040	K_id1022408	5.70	8.92	7.92	BRRI dhan28
Panicle Length	3	*qPL3.1*	30.18	K_id3003697	K_id3005956	3.02	7.13	-0.33	CSR28
No Filled Spikelets	10	*qNFS10.1*	75.93	K_id10005402	K_id10006100	4.16	12.82	-101.59	CSR28
No Unfilled Spikelets	3	*qNUFS3.1*	97.18	K_id3010094	K_id3011015	3.14	6.39	-78.16	CSR28
Percent Filled Spikelets	3	*qPFS3.1*	107.18	K_id3011015	K_id3014005	3.58	5.47	7.23	BRRI dhan28
	10	*qPFS10.1*	75.93	K_id10005402	K_id10006100	4.35	10.71	-7.90	CSR28
Grain Yield	10	*qGY10.1*	75.93	K_id10005402	K_id10006100	4.24	14.49	-2.08	CSR28
Na-K Ratio	1	*qNK1.1*	111.40	K_id1012666	K_id1015445	14.60	2.00	-0.02	CSR28
	1	*qNK1.1*	148.40	K_id1021040	K_id1022408	9.67	1.83	0.01	BRRI dhan28
	2	*qNK2.1*	117.27	K_id2012042	K_id2012908	11.32	2.18	0.04	BRRI dhan28
	2	*qNK2.2*	136.27	K_id2014452	K_id2014932	8.94	2.02	-0.02	CSR28
	5	*qNK5.1*	41.74	K_id5003638	K_id5005055	9.13	1.93	0.37	BRRI dhan28
	5	*qNK5.2*	48.74	K_id5005055	K_id5006615	9.63	2.09	0.37	BRRI dhan28
	12	*qNK12.1*	11.86	K_id12001224	K_id12001996	4.18	0.49	-0.07	CSR28

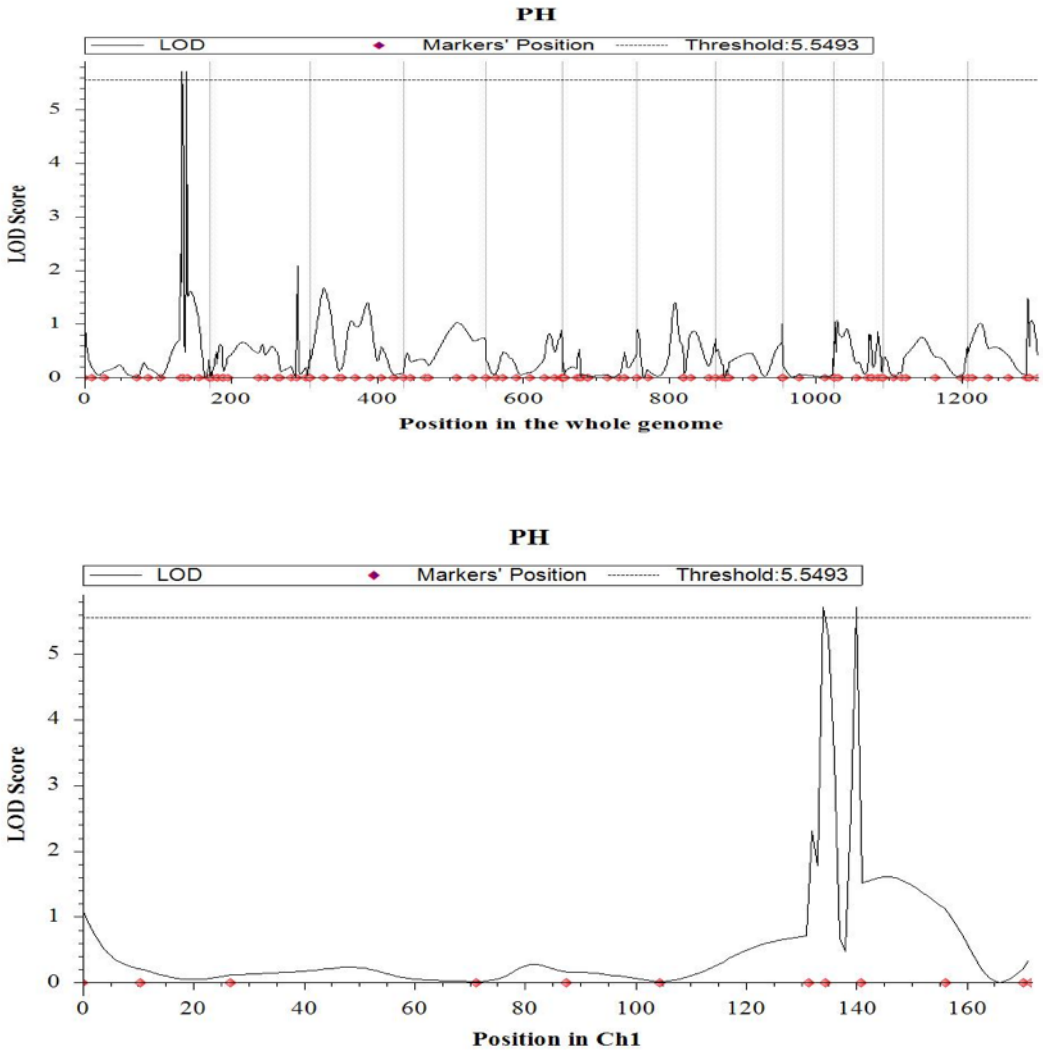


Figure 3.39. Chromosome locations of QTLs for plant height under salinity stress for 190 BC_1F_2 population from the cross, CSR28 x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes and (b) chromosome 1. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

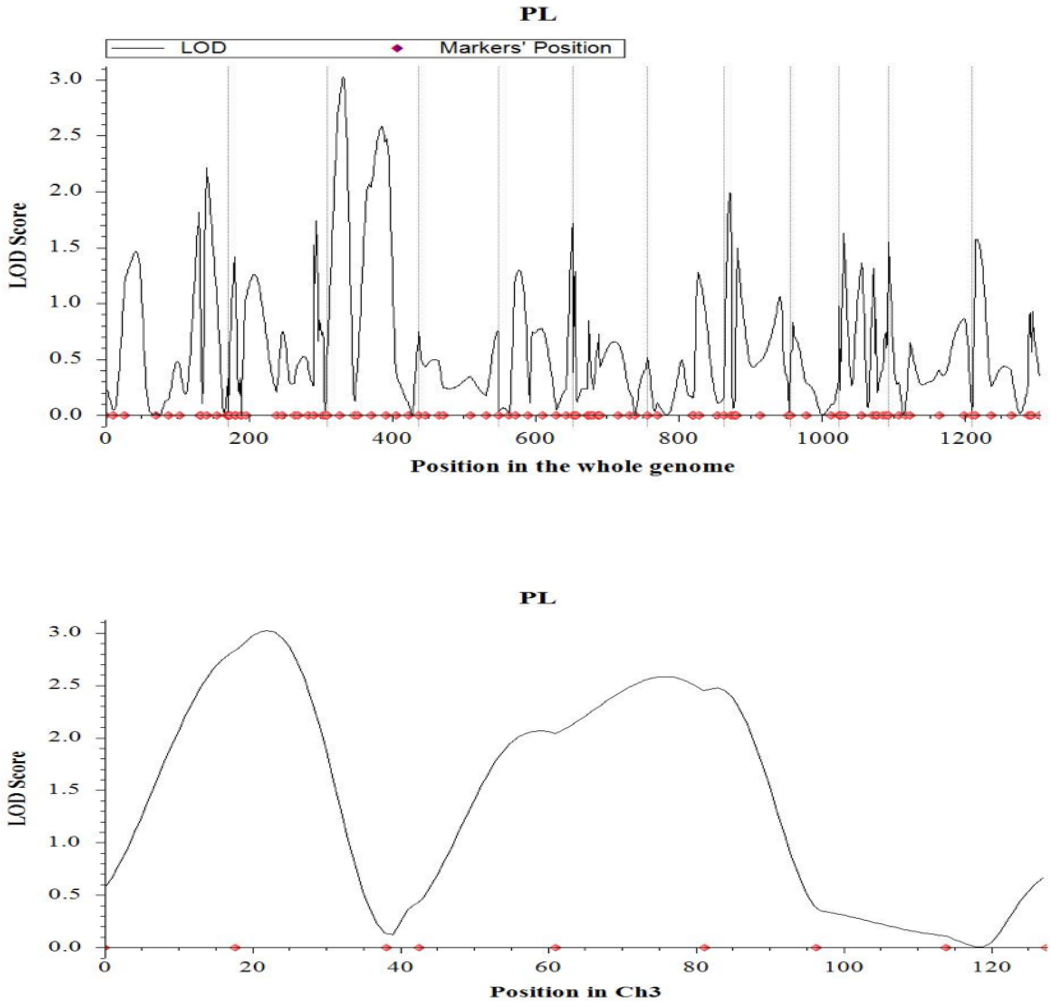


Figure 3.40. Chromosome locations of QTLs for panicle length plant under salinity stress for 190 BC_1F_2 population from the cross, CSR28 x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes and (b) chromosome 3.

3.4.4.4. Number of Filled and Unfilled Spikelets

A significant QTL ($p<0.05$) based on 1000 permutation was identified for number of filled spikelets on the long arm of chromosome 10 with a LOD value of 4.16. This QTL was mapped at 75.93 cM that can explain 12.82% phenotypic variation (Table 3.6, Figs. 3.38 and 3.41). Similarly, one QTL was found for number of unfilled spikelets on the long arm of

chromosome 3 with a LOD value of 3.14 and can explain 6.39% phenotypic variation (Table 3.6, Fig. 3.42). Both the QTLs (*qNFS10.1* and *qNUFS3.1*) exhibited negative additive effect indicated that the tolerant parent, CSR28 was responsible for the allele contribution.

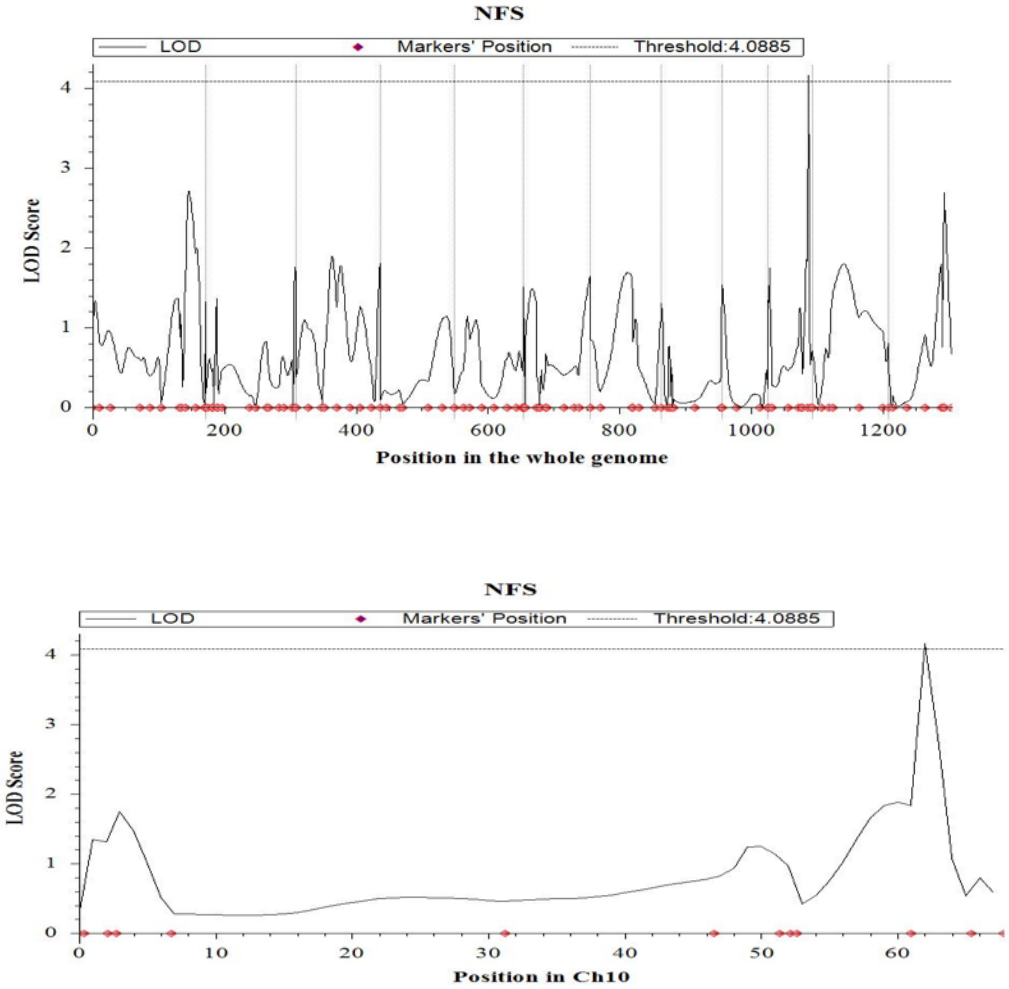


Figure 3.41. Chromosome locations of QTLs for number of filled spikelets under salinity stress for 190 BC_1F_2 population from the cross, CSR28 x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes and (b) chromosome 10. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

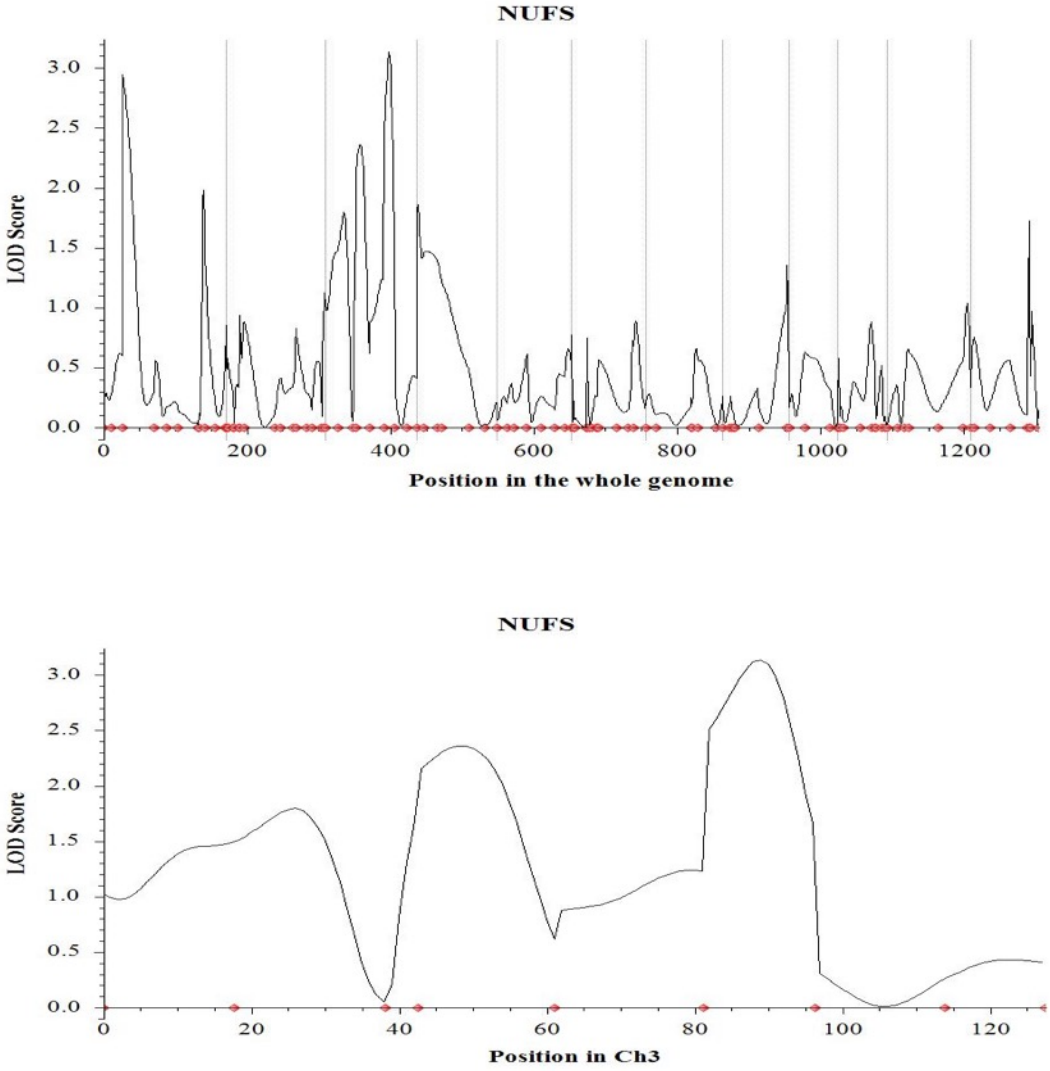


Figure 3.42. Chromosome locations of QTLs for number of unfilled spikelets under salinity stress for 190 BC_1F_2 population from the cross, CSR28 x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes and (b) chromosome 3.

3.4.4.5. Percent Filled Spikelets

Two QTLs were identified for the percent filled spikelets on the long arm of chromosomes 3 and 10 (Table 3.6, Figs. 3.38 and 3.43) under salinity stress at the reproductive stage of rice. The QTL on chromosome 10 (*qPFS10.1*) was significant ($p<0.05$) based on 1000 permutations and mapped at 75.93 cM with LOD value of 4.35 that explained 10.7% phenotypic variation. The additive effect was negative for *qPFS10.1* indicated that tolerant parent; CSR28 was responsible for this QTL.

3.4.4.6. Grain Yield

Only one significant ($p<0.05$) QTL, *qGY10.1* was identified for grain yield on the long arm of chromosome 10 with a LOD value of 4.24 based on 1000 permutations. This QTL was mapped at 75.93 cM that can explain 14.49% phenotypic variation (Table 3.6, Figs. 3.38 and 3.44). The additive effect was negative indicated that tolerant parent; CSR28 was responsible for this QTL.

Interestingly the QTLs for the number of filled spikelets (*qNFS10.1*), percent filled spikelets (*qPFS10.1*) and grain yield (*qGY10.1*) were mapped at the same position (75.93 cM) on the long arm of chromosome 10 and tolerant parent (CSR28) was responsible for contributing positive allele of these QTLs (Table 3.6, Fig, 3.38).

3.4.4.7. Sodium-Potassium (Na^+-K^+) Ratio

Seven QTLs were identified for Na-K ratio on chromosomes 1, 2, 5 and 12 (Table 3.6, Fig. 3.45). Among them, two QTLs (*qNK1.1* and *qNK1.2*) were identified on the long arm chromosome 1with LOD value of 14.6 and 9.67, and were mapped at 111.40 cM and 148.40 cM, respectively. Both the QTLs were contributed from CSR28. Besides chromosome 1, two more QTLs were found on chromosome 2 (*qNK2.1* and *qNK2.2*) with 11.32 and 8.94 LOD value and that explains 2.18% and 2.02% phenotypic variations, respectively. They were mapped at 117.27 cM and 136.27 cM and were contributed from the sensitive parent BRRI dhan28 and tolerant parent CSR28, respectively. In addition, two QTLs contributed from the sensitive parent (BRRI dhan28) were mapped on the short arm of chromosome 5 (*qNK5.1* and *qNK5.2*) at 41.74 cM and 48.74 cM with the LOD value of 9.13 and 9.63 that explained 1.93% and 2.09% phenotypic variations, respectively. The last QTL, *qNK12.1* was identified on the short arm of chromosome 12 with LOD value of 4.18 and were mapped at 11.86 cM. The tolerant parent, CSR28 parent contributed the alleles for this QTL.

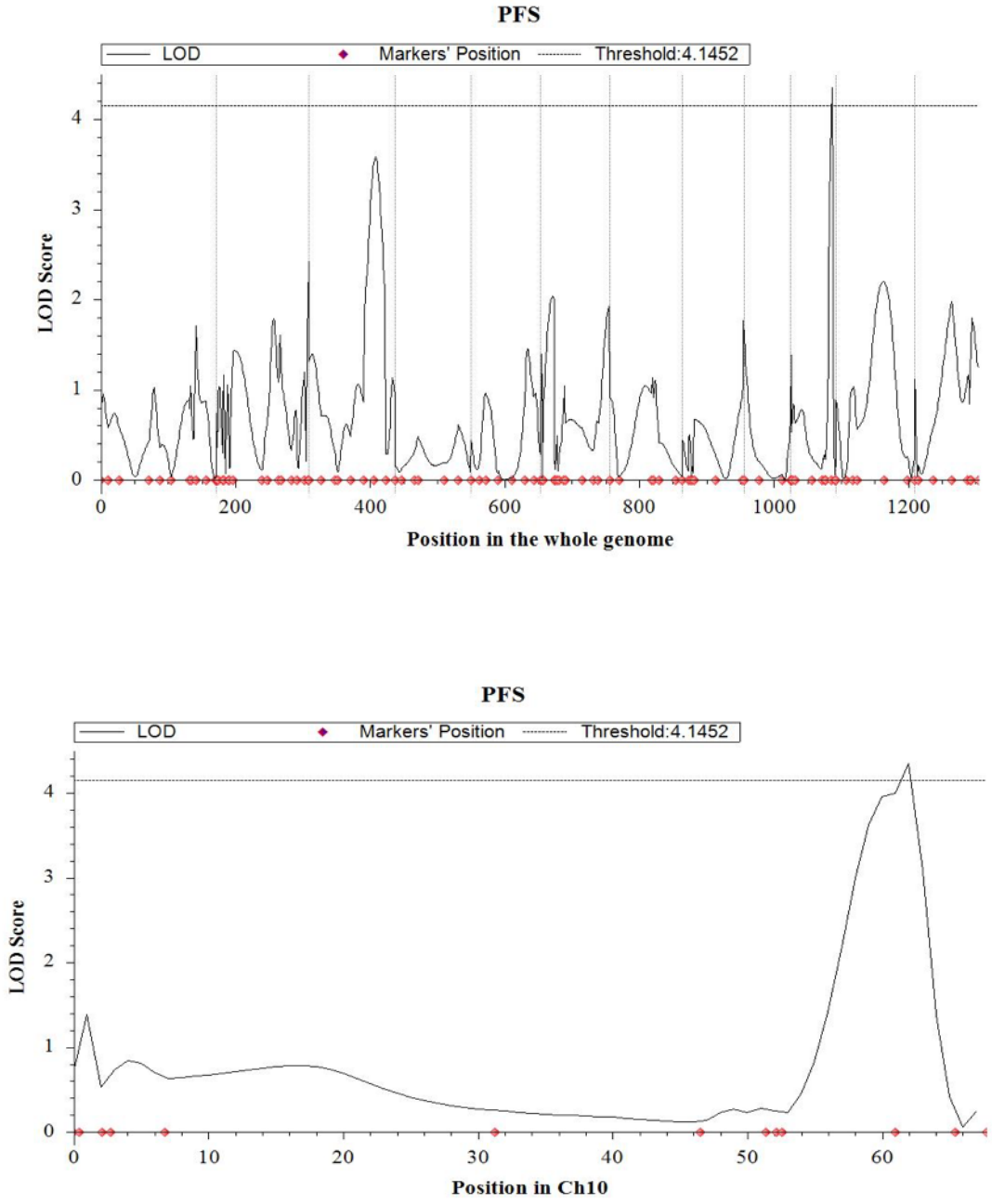


Figure 3.43. Chromosome locations of QTLs for percent filled spikelets under salinity stress for 190 BC_1F_2 population from the cross, CSR28 x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes and (b) chromosome 10. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

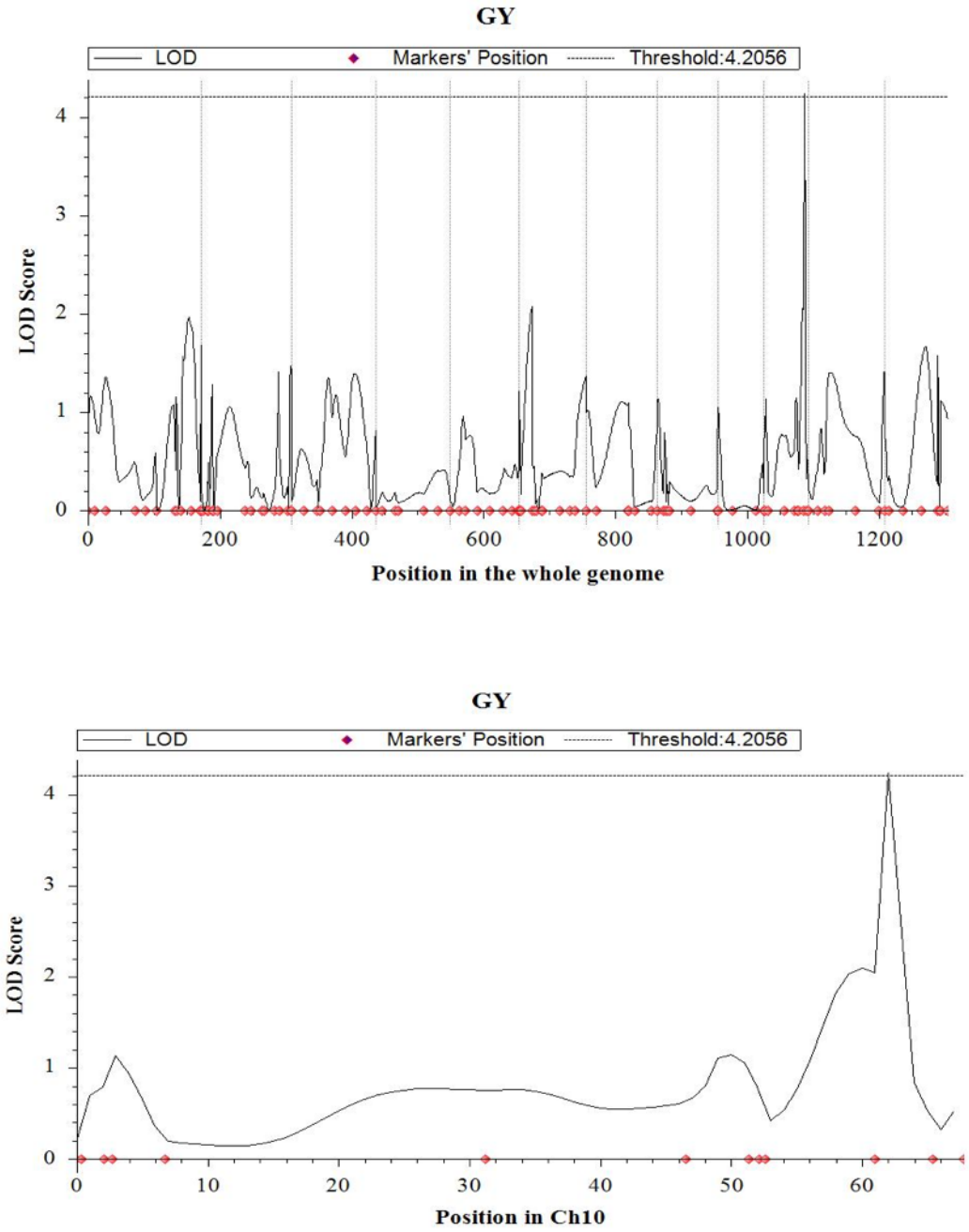


Figure 3.44. Chromosome locations of QTLs for grain yield under salinity stress for 190 BC_1F_2 population from the cross, CSR28 x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes and (b) chromosome 10. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

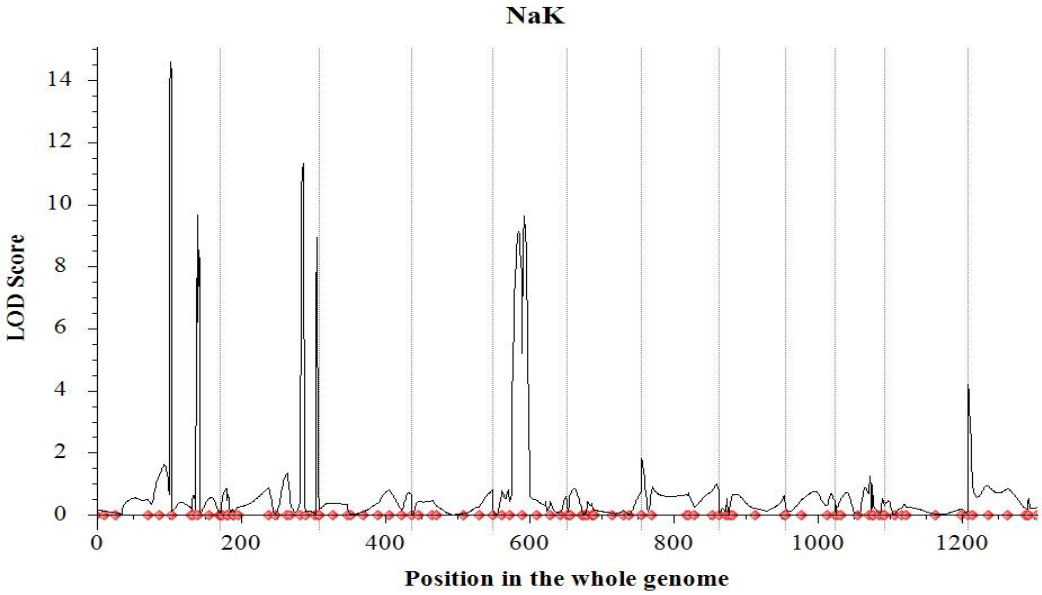


Figure 3.45. Chromosome locations of QTLs for Na-K ratio under salinity stress for 190 BC_1F_2 population from the cross, CSR28 x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping.

3.4.5. Comparison of New QTLs with Previous QTLs

Semi-dwarf 1 gene (*sd-1*) is the major gene of rice that revolutionized and significantly increased the yield of rice throughout the Asia during 1960s and onward. The phenotype of this gene is dwarfism which was the result from the deficiency of plant growth hormone, GA in the elongating stem (Sasaki et al., 2002). The location of this gene was on the long arm chomosome 1 and was mapped at 149.1 cM or 40.1 Mb. The QTLs identified for plant height in this study, *qPH1.1* (35.6 Mb). and *qPH1.2* (37.1) were mapped very near to the novel *sd-1* gene on the long arm of chromosome 1.

Similar QTLs for plant height were observed by Haque et al., (2020); Mondal et al., (2019); Hossain et al., (2015) and Mohammadi et al., (2013). Haque et al., (2020) identified three QTLs for plant height on chormosome 1, 3 and 5. The QTL, *qPH.1@ 215* was mapped

on the long arm of chromosome 1 at 215 cM and tolerant parent, Horkuch contributed positive allele for this QTL. Although the identified QTL in this study was also on chromosome 1, it was located (around 41 cM) far from the QTL identified in this study. In addition, Mondal et al., (2019) identified one QTL on chromosome 1 for plant height by using composite interval mapping (CIM) and tolerant parent (BRRI dhan47) was responsible for this QTL. Hossain et al., (2015) evaluated 218 F_2 individuals of Cheriviruppu (highly salt tolerant at both seedling and reproductive stages) x Pusa Basmati-1 (PB1) (sensitive at both seedling and reproductive stages) at the reproductive stage using salinized water (EC 10 dS/m) and reported three significant QTLs on chromosomes 1, 4 and 7 for plant height. Cheriviruppu alleles contributed to the tallness of plants for all the QTLs. The long arm of chromosiome 1 contained a major QTL for plant height, *qPH-1.1s*, with 47.2% phenotypic variation. While, Mohammadi et al., (2013) obtained QTL for plant height on chromosomes 1 (*qPH1.1s*), 2 (*qPH2.1s*), 3 (*qPH3.1s*) and 7 (*qPH7.1s*) using CIM with 232 F_2 population derived from the cross SADRI (tolerant at reproductive stage) and FL478 (sensitive at reproductive stage) using 6-8 dS/m salinity stress. The loci had R^2 value ranging from 6.6 to 17.0 %. The QTL, *qPH1.1*s detected near the marker RM212 located on the long arm of chromosome 1 with the highest LOD value (9.4); the alleles from the tolerant increased plant height.

Number and percent filled spikelets are the most important traits for salinity tolerance particularly at the reproductive stage in rice and grain yield is the ultimate indicator for salinity tolerance. All of the traits are good indicators to determine a plant either salt tolerant or salt sensitive. In this study, one significant QTL ($p<0.05$) each for the three traits was detected on the long arm chromosome 10 (*qNFS10.1*, *qPFS10.1* and *qGY10.1*). The QTLs

were mapped at 75.93 cM and flanked by two markers, k_id10005402 (18.73 Mb) and k_id10006120 (19.83 Mb) and the tolerant parent; CSR28 contributed the alleles. Haque et al., (2020) also detected QTL for the number of filled grains on chromosome 10 but the position (58.48 cM) was different from the current study (around 17 cM beforehand than the current QTL). This may be due to the crossing of different parents with different salinity treatments.

A rice nuclear gene, *Rf-1*, that restores the pollen fertility was found on the chromosome 10 at 19.4 Mb (Komori et al., 2004) which was very near to the position (19.83 Mb) of the QTLs identified in this study for the traits: number of filled spikelets (*qNFS10.1*), percent filled spikelets (*qPFS10.1*) and grain yield (*qGY10.1*). *Rf-1* was distributed by the BT-type male sterile cytoplasm and used for the production of *japonica* hybrid varieties. It is the first restorer gene that reduces the expression of the cytoplasmic male sterility (CMS)-associated mitochondrial gene. *Rf-1* encodes a mitochondrially targeted protein containing 16 repeats of the 35-aa pentatricopeptide repeat (PPR) motif and recessive allele *rf-1* lacks one nucleotide in the coding region probably due to frame shift mutation resulting in a truncated protein (Komori et al., 2004).

Wang et al., (2006) found RF1A and RF1B; and Hu et al., (2012) identified Rf5 gene on chromosome 10 at 19.39 Mb, 19.51 Mb and 19.39 Mb, respectively. RF1A and RF1B are both targeted to mitochondria and can restore male fertility by blocking ORF79 production which was an abnormal mitochondrial open reading frame that cause male sterility in CMS lines and transgenic rice plants. ORF79 was blocked via endonucleolytic cleavage by RF1A or degradation of dicistronic B-*atp6*/*orf79* mRNA by RF1B. While the fertility restorer gene, Rf5 was cloned to understand the fertility restoration mechanism in rice. The QTLs for

number of filled spiekelets (*qNFS10.1*), percent filled spikelets (*qPFS10.1*) and grain yield (*qGY10.1*) identified in this study were very near to both the genes (19.83 Mb). Wang et al., (2012)also found *Osj10BTF3* gene on chromosome 10 at 18.69 Mb resulting in significant plant dwarfism of about 25% to 52% and complete pollen abortion due to inhibition of *Osj10BTF3*. *BTF3* was a basal transcriptional factor that involved in transcription initiation, translational regulation and protein localization in many eukaryotic organisms. Initially, the expression of this gene was constitutive and modulated by salinity, heat and exogenous phytohormone stress. Besides, QTL for 1000 grain weight was also identified on chromosome 10 from the crosses Zhenshan 97B x Milyang 46 was mapped in between 19.73 Mb to 22.30 Mb with LOD value of 7.98 (Zhuang et al., 2001).

3.5. Summary and Conclusion

In this study, 624 BC_1F_2 population derived from the cross between a sensitive variety, BRRI dhan28, and a salt-tolerant variety, CSR28, were evaluated under a salinity stress of EC 10 dS/m to identify quantitative trait loci (QTLs) for salinity tolerance at the reproductive stage of rice. Continuous 20 days salt stress was applied at the reproductive stage to 435 BC_1F_2 progenies and the remaining 189 progenies were grown under non-stress (control) condition. Among 435 progenies of CSR28 x BRRI dhan28, 46% plants were classified as tolerant, and the rest as sensitive based on their SES score and grain yield. About 45% each of the extremely tolerant and highly sensitive progenies were used for QTL mapping. The findings and conclusions drawn from this study are summarized below.

The agronomic and yield related traits evaluated for salinity stress were plant height (PH), productive tillers (PT), panicle length (PL), number of filled spikelets (NFS), number

of unfilled spikelets (NUFS), percent filled spikelets (PFS), percent unfilled spikelets (PUFS) and grain yield (GY). In addition, a physiological trait, sodium-potassium (Na^+/K^+) ratio was also evacuated. Almost all the traits differed significantly under salinity stress over those obtained under non-stress condition, except the grain yield of CSR28 x BRRI dhan28. But all the traits including the grain yield of the tolerant and sensitive progenies grown under salinity stress differed significantly. Yield reduction between tolerant progenies of CSR28 x BRRI dhan28 grown under salinity stress and no-stress condition was 15.8%.

The correlation analysis indicated positive and significant ($p<0.001$) correlation between grain yield and the number of filled spikelets, percent filled spikelets and productive tillers and significantly negative ($p<0.001$) correlation with the SES score and percent unfilled spikelets of the BC_1F_2 progenies derived from this cross.

A total of 15 QTLs related were identified through inclusive composite interval mapping (ICIM) of the cross, CSR28 x BRRI dhan28 using IciMapping software 4.2. The QTLs were identified in all the traits except in the number of productive tillers per plant. Total size of the genetic linkage map was 1428.69 cM, constructed using 116 SNP markers. Among the 15 QTLs; two QTLs each were identified for plant height and percent filled spikelets, and one each for panicle length, number of filled and unfilled spikelets and grain yield. The tolerant parent, CSR28 contributed by additive effects to the QTLs for plant height, panicle length, number of filled and unfilled spikelets, percent filled spikelets and grain yield. But both the parents (BRRI dhan28 and CSR28) contributed alleles for plant height and percent filled spikelets. All the data were statistically confirmed with permutation analysis of 1000 times at 5% level of significance. Despite a few QTLs identified, two QTLs

for plant height and one each for number of filled spikelets, percent filled spiklets and grain yield were statistically confirmed for the progenies of CSR28 x BRRI dhan28.

The QTLs identified in this study for reproductive-stage salt tolerance need to be fine mapped before they can be directly used to accelerate marker-assisted selection in future breeding programs to increase selection efficiency. The identification of the genes constituting these major QTLs would help to understand the molecular mechanisms.

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4. MAPPING QTLs FOR REPRODUCTIVE STAGE SALINITY TOLERANCE IN RICE USING BC_1F_2 POPULATION OF HASAWI x BRRI dhan28

4.1. Introduction

Rice (*Oryza sativa* L.) is the major staple for almost half of the world's population. Sustained rice production is, therefore essential for food security of a large community across the globe. Unfavorable environmental conditions such as salinity, drought, heat, and submergence pose a huge threat to rice productivity and challenge future food security. Among all the abiotic stress, salinity is the second most prevalent problem affecting rice productivity worldwide (Flowers, 2004) and posing a serious threat to rice-based farming system especially in the fragile coastal zone. Aside from coastal areas, about 20% irrigated and 8% of rainfed agricultural land (Asif et al. 2018) and about one-third of the irrigated rice growing areas are affected by salinity (Prasad et al., 2000). The salt affected areas are expected to increase due to the adverse effect of climate change and sea level rise (IPCC, 2019). Therefore, improving the productivity of crops, especially the main staple, in salt-affected areas of the world is considered essential to meet the increasing food demand and sustained food security.

Salinity tolerance is a complex trait and recognized to multiple mechanisms. Salt injury could be overcome by extruding Na^+ from the cytoplasm which depends on the mechanisms of Na^+ extrusion from roots, unloading Na^+ from the xylem, and by sequestration of Na^+ into vacuoles (Ismail and Horie, 2017). It is not only confined with the Na^+ exclusion or sequestration, rather presence of cytosolic K^+, osmolytes, compatible

solutes play an important role in salinity tolerance. Understanding the proper mechanisms will help to identify the genetic materials.

Salinity affects plant growth during all developmental stages. Rice is relatively tolerant to salinity stress during germination, active tillering and at maturity; but is very sensitive at the early seedling and reproductive stages (Singh et al., 2007; Singh and Flowers 2010). The reproductive stage is crucial as it ultimately translates grain yield. Although salinity at the reproductive stage depresses grain yield much more than the vegetative stage but there are few studies in rice for salinity tolerance at the reproductive stage due to the difficulty of achieving reliable stage-specific phenotyping techniques (Ahmadizadeh et al., 2016).

Besides, the salinity rice breeding progress is slower because of genetic complexity and environmental factors as salinity tolerance is governed by many genes that exhibit the polygenic nature. The genetics behind salinity tolerance can be revealed by using quantitative trait locus (QTLs) analysis (Gimhani et al., 2016; Negrão et al., 2011). Recent advances in molecular marker technology have enabled the dissection of the molecular mechanisms of salinity tolerance to identify major-effect QTLs (Munns 2005; Tuberosa and Salvi 2005; Thomson et al., 2010, 2012; Thomson, 2014; Hossain et al., 2015). Single nucleotide polymorphisms (SNPs) are the markers of choice for most high throughput genotyping applications because they are ubiquitous in eukaryotic genomes, cost-effective to assay using automated platforms, and biallelic in nature, which is useful for allele calling, data analysis and data-basing (Thomson et al., 2017).

Identifying useful alleles and introgress those alleles into mega variety is a key to successful breeding approach for sustaining productivity. The Aus landrace "Hasawi," originated from eastern Saudi Arabia, is found to have higher Na^+ exclusion and early seedling vigor (Thomson et al., 2010; Rahman et al., 2017; Bizimana et al., 2017). It is a highly salt tolerant genotype and characterized by its strong adaptability to soil salinity and drought. But it has some undesirable characteristics like susceptibility to lodging, delayed maturity, and photoperiod sensitivity (Bimpomg et al., 2014). Thomson et al. (2010) suggested that high level of polymorphism could be observed in the progenies derived by crossing Hasawi and Indica cultivars. Bimpong et al. (2014) reported four grain yield-enhancing QTLs (qPH8, qDTF8, qTN8, and qTN8) from Hasawi at reproductive stage under salinity stress. On the other hand, BRRI dhan28, an indica cultivar is a Bangladeshi mega variety has all the desirable characteristics for higher productivity except sensitive to salinity. In this study, BC_1F_2 mapping population developed from the cross between Hasawi and BRRI dhan28 at the International Rice Research Institute (IRRI) for QTL analysis and a high-density SNP linkage map was used to identify QTLs associated with salinity related traits.

4.2. Objective

The general objective of this study was to identify quantitative trait loci (QTLs) for salinity tolerance at the reproductive stage of rice plant using BC_1F_2 population derived from the salt tolerant variety, Hasawi (Saudi Arabian variety) and a salt sensitive Bangladeshi variety, BRRI dhan28. The specific objectives were:

a. To screen the BC_1F_2 population along with their parents under salinity stress for establishing relative importance of different traits associated with the reproductive stage salinity tolerance in rice.

b. To genotype the BC_1F_2 population for mapping the large effect QTLs responsible for tolerance to salt-stress at the reproductive stage of rice.

c. To identify candidate diagnostic markers linked to the QTLs conferring salinity tolerance from the tolerant parents.

d. To identify the candidate genes.

4.3. Materials and Methods

A salt-tolerant Saudi Arabian variety, Hasawi and a salt-sensitive Bangladeshi rice variety, BRRI dhan28 was used as the parents to develop a mapping population. The BC_1F_2 QTL mapping populations was generated by crossing the salt-tolerant donor parents (Hasawi) with the salt sensitive parent (BRRI dhan28). The parents belong to sub-species Indica. The cross, Hasawi x BRRI dhan28 were developed at the rice breeding platform of the International Rice Research Institute (IRRI), Philippines crossing program to generate F_1 and then backcross to recurrent parent, BRRI dhan28 to produce the BC_1F_2 progenies.

In this experiment, 588 BC_1F_2 lines derived from the cross, Hasawi x BRRI dhan28 were evaluated in the wet season (July-December 2018) under control (n=153 progenies) salt stress of EC 10 dS/m (n=435 progenies) in the concrete tank at IRRI, Philippines.

The methodology followed in this experiment is discussed detailed in Chapter 3. The phenotyping methodology followed in this experiment was same as that discussed in Chapter 3 from Section 3.2.1 to Section 3.2.7. And methodology for DNA Extraction included under Section 3.2.8.1, and QTL mapping for salinity tolerance in Section 3.2.9.

The only exception was the methodology used for Molecular Characterization (Section 3.2.8). In this experiment, SkimGBS platform was used for QTL mapping (instead of KASP genotyping followed in the first experiment), methodology of which is given below.

Skim genotyping by sequencing (GBS) is a novel method which is used to determine the differences in the genetic makeup of individuals (Golicz et al., 2015). It is a combination

of genotyping and next-generation sequencing. Basically, genotyping is a process of determining the differences in the genetic makeup (genotype) of individuals by examining their DNA either having insertions, deletions, or single nucleotide polymorphisms (SNPs). SNP discovery is usually performed by resequencing of the parental individuals followed by read alignment to the available reference sequence and SNP calling. Typically, skim-based SNP genotyping involves resequencing of multiple individuals followed by alignment of the reads to the reference sequence. For each of the individuals, reads mapping to the known SNP positions are inspected and the SNP alleles are recorded. Using this approach, genotype maps for the entire genomes can be understood and making it possible to detect that which part of the genome was inherited from each of the parental individuals. In this experiment, 192 BC_1F_2 population from the crosses between Hasawi x BRRI dhan28 was used for skim-based resequencing and PR106::IRGC53418-1 used for alignment as it has higher quality of assembly.

4.4. Results and Discussion

4.4.1. Responses of the Parental Lines and BC_1F_2 Progenies to Salt Stress

A total of 588 BC_1F_2 genotypes from the cross, Hasawi x BRRI dhan28 were included in this study, in which salt stress of EC 10 dS/m was imposed on 435individuals and 153 individuals were grown under control (no-stress) condition. Of the 435 progenies, 124 individuals were classified as tolerant and 311 as sensitive based on their SES score and yield.

Selective genotyping method was used to identify the most tolerant and sensitive progenies. After screening based on grain yield and SES, the number of extremely tolerant and highly sensitive BC_1F_2 progenies from this cross was 78 and 112, respectively. The effect of salinity on agronomic and physiological characters is discussed below:

4.4.1.1. Plant Height

The plant height of the BC_1F_2 progenies of Hasawi x BRRI dhan28 varied from 37.0 to 180.0 cm under salinity stress of 10 dS/m at the reproductive stage (Fig. 4.1, Table 4.1). The interquartile range was 59.5 cm under stress condition and the range was highly variable among the progenies of Hasawi x BRRI dhan28 (Fig. 4.2). Among the families in Hasawi x BRRI dhan28, the plant families IR131853-4, 5 and 7 produced the tallest and IR131853-6 had shortest plant height under salinity stress condition (Fig. 4.3).

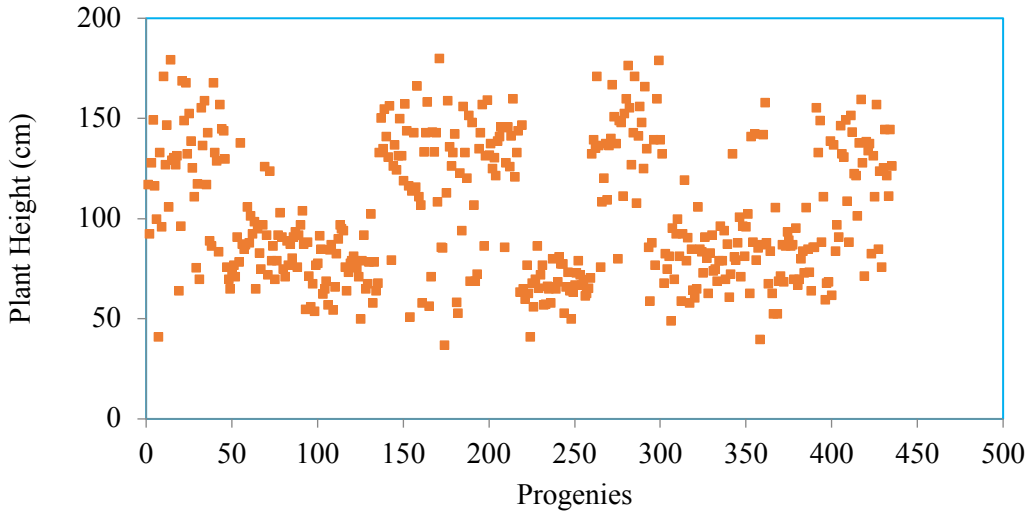
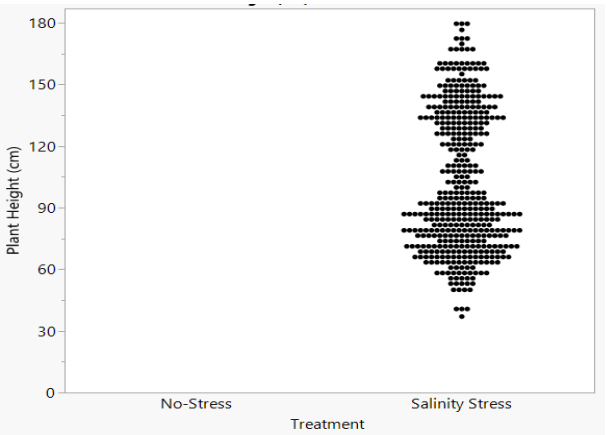


Figure 4.1. Scattered plot of the plant height of the salinity-stressed BC_1F_2 individuals of rice from the cross, Hasawi x BRRI dhan28.

a. Scatter plot



a. Box plot

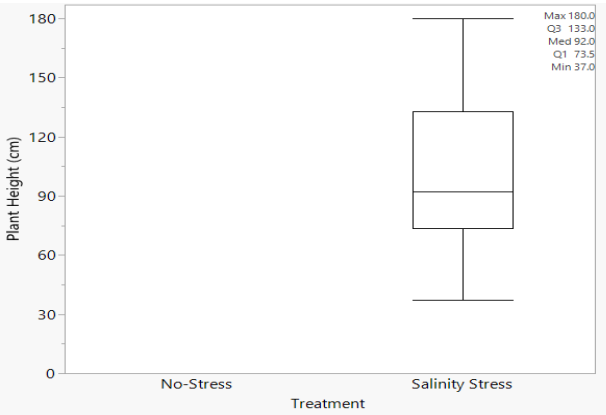


Figure 4.2. Scattered plot (a) and box (b) plots of the plant height of BC_1F_2 individuals of Hasawi x BRRI dhan28 under salinity stress at reproductive stage of rice.

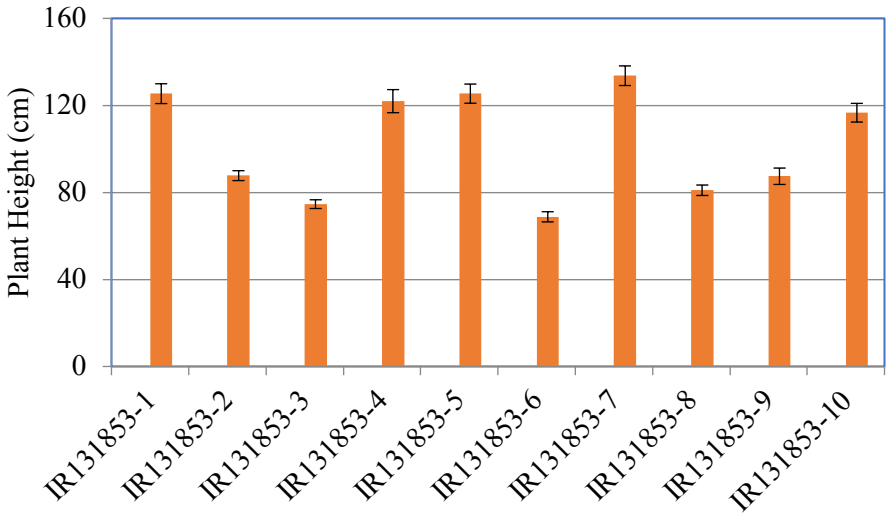


Figure 4.3. Mean plant height of the rice families of Hasawi x BRRI dhan28 progenies under salinity stress. Vertical and capped bar indicates standard error of the mean plant height of 42 to 46 plants per family.

The plant height of the tolerant and sensitive parents was 124.0 cm and 90.0 cm, respectively under control condition (Table 4.1, Fig. 4.4). Under selective genotyping, the mean plant height of the tolerant and sensitive progenies of Hasawi x BRRI dhan28 differed significantly under salinity stress (Fig. 4.4, Table 4.2). The plant height of the tolerant and sensitive progenies was 116.3 cm and 98.3 cm, respectively. Reduction in plant height of sensitive genotypes was 15.5% over tolerant genotypes (Table 4.1).

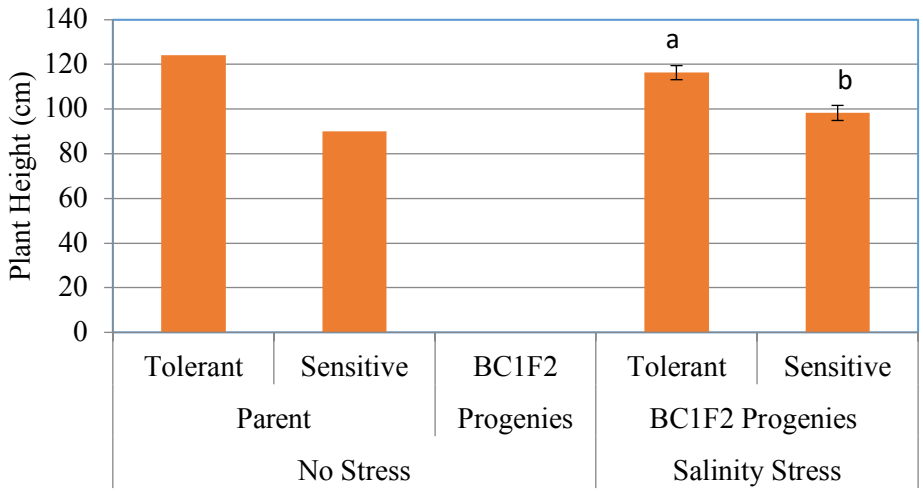


Figure 4.4. Mean plant height of tolerant and sensitive parents under no-stress and their BC_1F_2 progenies under salinity stress at reproductive stage of rice. Vertical and capped bar indicates standard error of the mean plant height of 78 tolerant and 112 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

Table 4.1. Descriptive statistics of the BC_1F_2 progenies of Hasawi x BRRI dhan28.

Traits	Parents		BC_1F_2 Progenies of Hasawi x BRRI dhan28							Decrease over No-Stress (%)		Decrease Sensitive over Tolerant (%)
	Hasawi	BRRI dhan28	Mean	Range	Skew-ness	SE	No-Stress	Tolerant	Sensitive	Tolerant	Sensitive	
Plant Height (cm)	124	90	102.02	37-180	0.36	1.61		116.31	98.27			15.51
Productive Tiller (no/plant)	18	25	12.65	1-39	0.44	0.33		17.53	10.49			40.14
Panicle Length (cm)	20.7	27	21.20	7.5-31.5	5.22	0.23		23.14	20.38			11.91
Filled Spiketets (no/plant)	1708	2349	307.59	0-2115	1.89	16.54	637.48	735.72	123.59	-15.41	80.61	83.20
Unfilled Spiketets (no/plant)	436	617	633.40	17-3316	1.40	25.39	448.87	793.55	592.84	-76.79	-32.07	25.29
Filled Spikelets (%)	79.66	79.20	29.36	0-84.7	0.42	0.95	59.14	49.69	20.64	15.98	65.10	58.46
Grain Weight (g/plant)	47.97	54.98	5.85	0-43.49	2.11	0.34	16.48	15.06	2.13	8.64	87.07	85.85
Na-K Ratio	1.927	3.105	0.86	0.027-14.5	4.46	0.08		0.52	1.18			-128.56

Table 4.2. ANOVA of the BC_1F_2 genotypes of Hasawi x BRRI dhan28 (values with the same letter in a column are not significantly different at 5% level of significance).

Treatment	BC_1F_2 Genotypes/ Progenies	Plant Height (cm)	Productive Tillers (no/plant)	Panicle Length (cm)	Filled Spikelets (no/plant)	Unfilled Spikelets (no/plant)	Filled Spikelets (%)	Unfilled Spikelets (%)	Grain Yield (g/plant)	Na-K Ratio
Selected Genotypes										
No-Stress	Control				637.5^{a}	448.9^{c}	59.1^{a}	40.9^{c}	16.5^{a}	
Salinity-Stress	Tolerant	116.3^{a}	17.5^{a}	23.1^{a}	735.7^{a}	793.6^{a}	49.7^{b}	50.3^{b}	15.1^{a}	0.52^{b}
	Sensitive	98.3^{b}	10.5^{b}	20.4^{b}	123.6^{b}	592.9^{b}	20.6^{c}	79.4^{a}	2.1^{b}	1.18^{a}
$LSD_{0.05}$		9.52	1.79	1.03	104.61	125.96	3.98	3.98	2.34	0.55
All Genotypes										
No-Stress (N)					637.5^{A}	448.9^{A}	59.1^{A}	40.9^{B}	16.5^{A}	
Salinity Stress (S)		102	12.7	21	307.6^{B}	633.4^{B}	29.4^{B}	70.6^{A}	5.9^{B}	
$LSD_{0.05}$					69.90	90.74	3.51	3.51	1.66	

4.4.1.2. Productive Tillers

The number of productive tillers of the BC_1F_2 progenies of Hasawi x BRRI dhan28 ranged from 1 to 39 under salinity stress condition (Fig. 4.5, Table 4.1). The interquartile range of the productive tillers for stress condition was 9 per plant (Fig. 4.6).

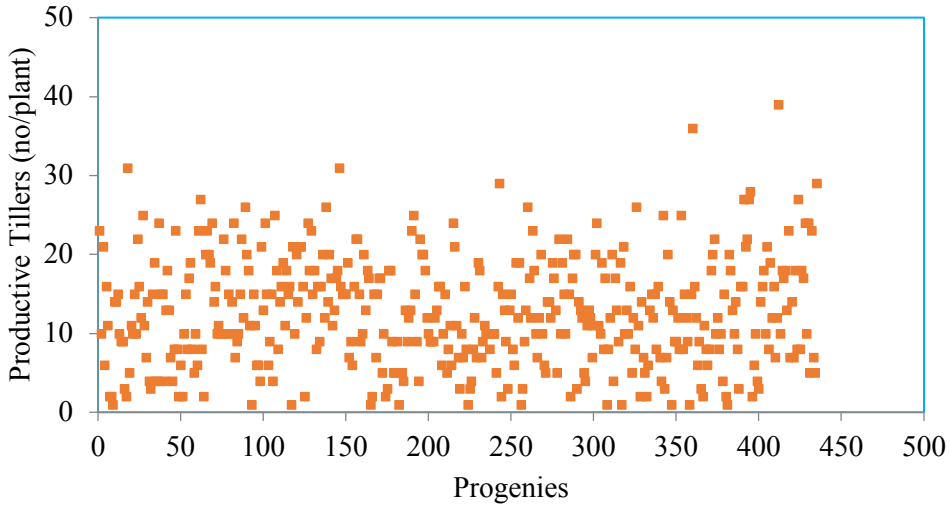
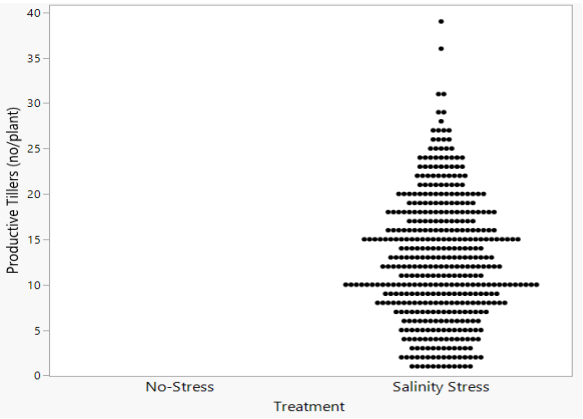


Figure 4.5. Number of productive tillers per plant of the BC_1F_2 individuals of the cross, Hasawi x BRRI dhan28 grown under salinity stress.

a. Scatter plot



b. Box plot

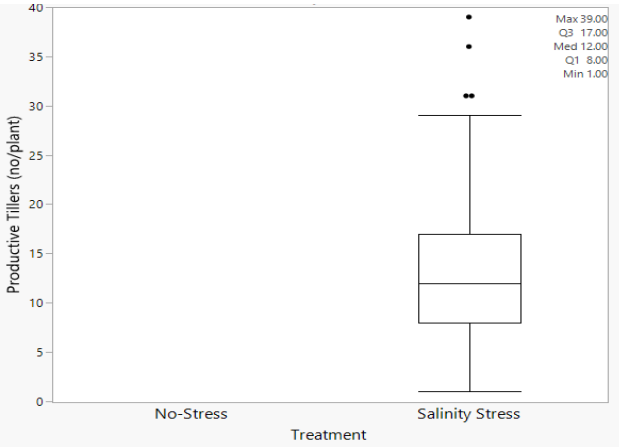


Figure 4.6. Scattered (a) and box (b) plots of the number of productive tillers per plant of BC_1F_2 individuals under salinity stress at reproductive stage of rice.

The variability in the number of productive tillers among the plant families from the cross, Hasawi x BRRI dhan28 is shown in Fig. 4.7. Among the families, IR131853-10 produced more productive tillers than other plant families under salinity stress.

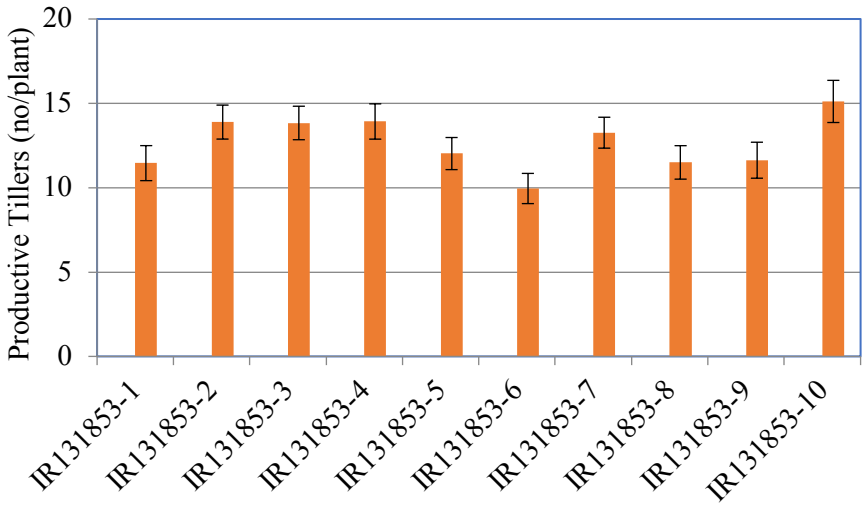


Figure 4.7. Mean number of productive tillers in rice families of Hasawi x BRRI dhan28 progenies under salinity stress. Vertical and capped bar indicates standard error of the mean productive tillers of 42-46 plants per family.

The number of productive tillers of the tolerant and sensitive parent was 18 and 25 tillers per plant under no-stress condition (Table4.1, Fig. 4.8). Under selective genotyping, the number of productive tillers per plant differed significantly between tolerant and sensitive individuals (Fig. 4.8, Table 4.2). The number of productive tillers for tolerant and sensitive progenies was 17.5 and 10.5 per plant, respectively. The reduction in productive tillers of sensitive genotypes was 40.1% over tolerant genotype of Hasawi x BRRI dhan28.

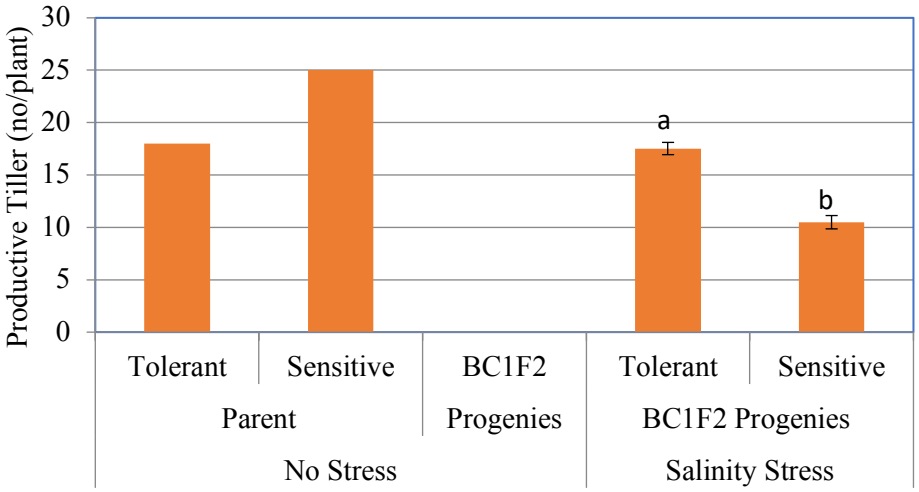


Figure 4.8. Mean productive tillers per plant of the tolerant and sensitive parents and their BC_1F_2 progenies under salinity stress at reproductive stage of rice. Vertical and capped bar indicates standard error of the mean productive tillers of 78 tolerant and 112 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

4.4.1.3. Panicle Length

The panicle length of the BC_1F_2 progenies of Hasawi x BRRI dhan28 varied from 7.5 cm to 31.5 cm under salinity stress (Fig. 4.9, Table 4.1). The interquartile range of the panicle length of the progenies was 4.9 under stress condition (Fig. 4.10).

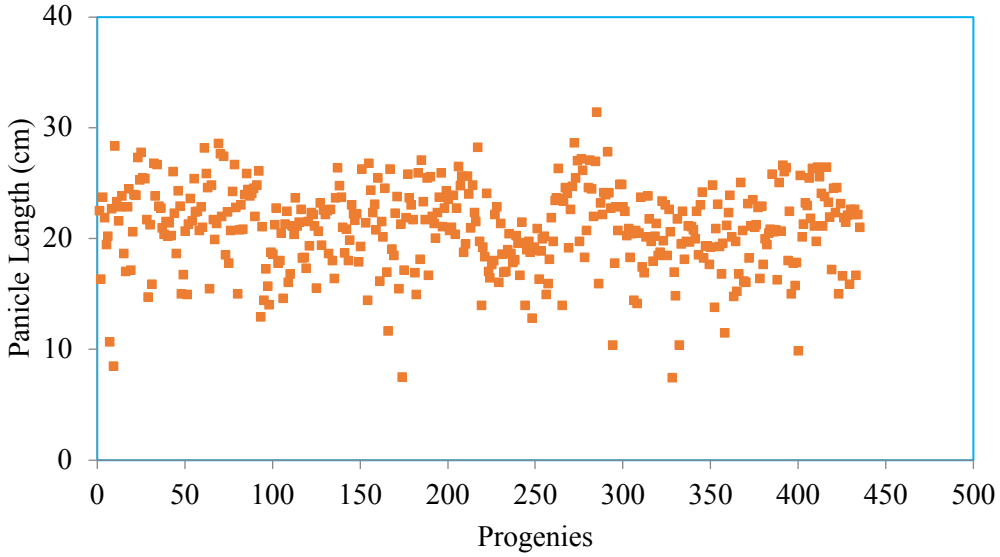
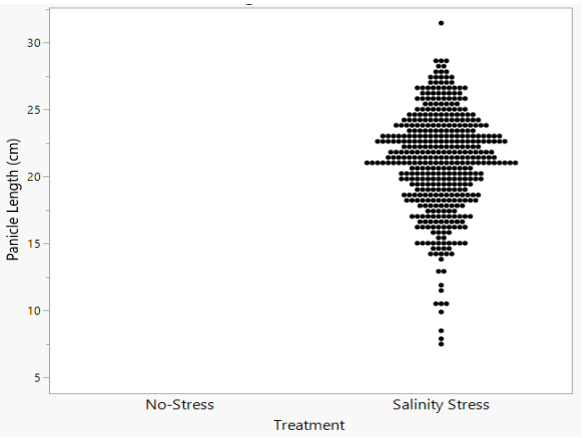


Figure 4.9. Panicle length of rice of the salinity-stressed BC_1F_2 individuals of the cross, Hasawi x BRRI dhan28.

a. Scatter plot



b. Box plot

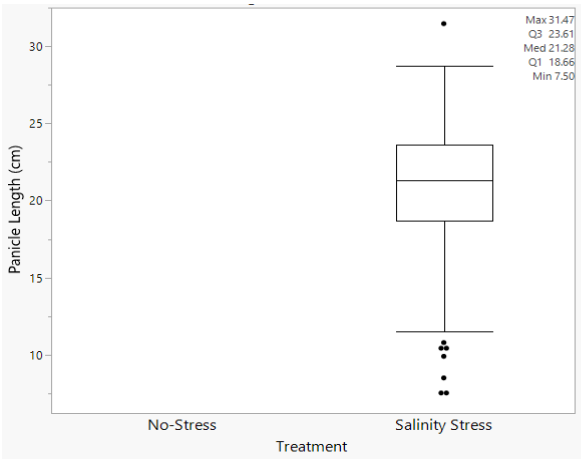


Figure 4.10. Scattered (a) and box (b) plots of the panicle length of BC_1F_2 individuals of Hasawi x BRRI dhan28 grown under salinity stress.

The panicle length of the plant families from Hasawi x BRRI dhan28 varied (Fig. 4.11) and among the families, highest panicle length was observed in IR131853-1 and IR131853-7 and the shortest panicles were found in IR131853-6 under salinity stress.

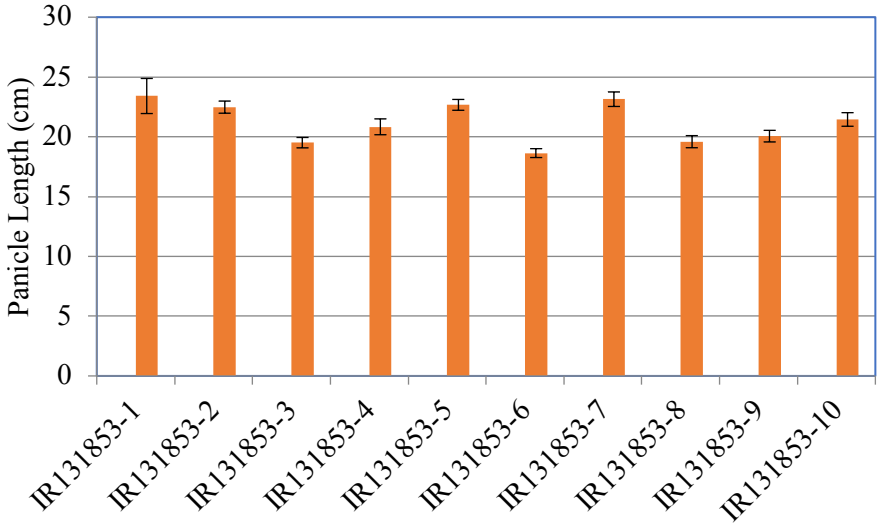


Figure 4.11. Mean panicle length of the rice families of Hasawi x BRRI dhan28 progenies under salinity stress. Vertical and capped bar indicates standard error of the mean panicle length of 42-46 plants per family.

The panicle length of the tolerant parent, Hasawi was 20.7 cm, while it was 27.0 cm for the sensitive parent, BRRI dhan28 under non-stress condition (Table 4.1; Fig. 4.12). Under selective genotyping, the panicle length of the progenies from this cross varied significantly between tolerant and sensitive individuals (Fig. 4.12, Table 4.2). The mean panicle length of tolerant and sensitive progenies of Hasawi x BRRI dhan28 was 23.1 cm and 20.4 cm, respectively under salinity stress. The reduction in panicle length of sensitive progenies was 11.9% over the tolerant progenies (Table 4.1, Fig. 4.12).

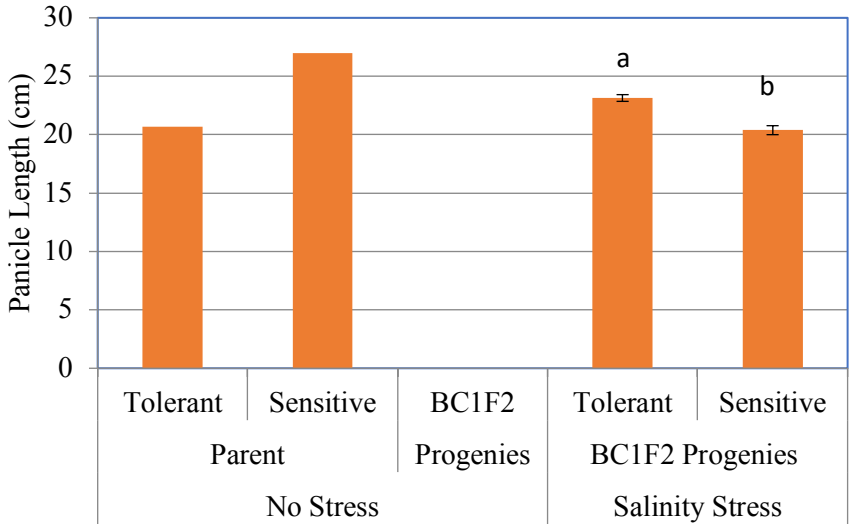


Figure 4.12. Mean panicle length of the tolerant and sensitive parents and their BC_1F_2 progenies under non-stress and salinity stress at reproductive stage of rice. Vertical and capped bar indicates standard error of the mean panicle length of 78 tolerant and 112 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

4.4.1.4. Number of Filled Spikelets

The number of filled spikelets of the tolerant parent, Hasawi was 1708 and sensitive parent, BRRI dhan28 was 2349 per plant under no-stress condition (Table 4.1). While the filled spikelets of BC_1F_2 progenies of Hasawi x BRRI dhan28 varied from 98 to 2950 under no-stress and from 0 to 2115 filled spikelets per plant under salinity stress (Fig. 4.13). Highly significant variation ($p<0.001$) in the number of filled spikelets was observed between the control and salinity treatment (Fig. 4.14, Table 4.2).

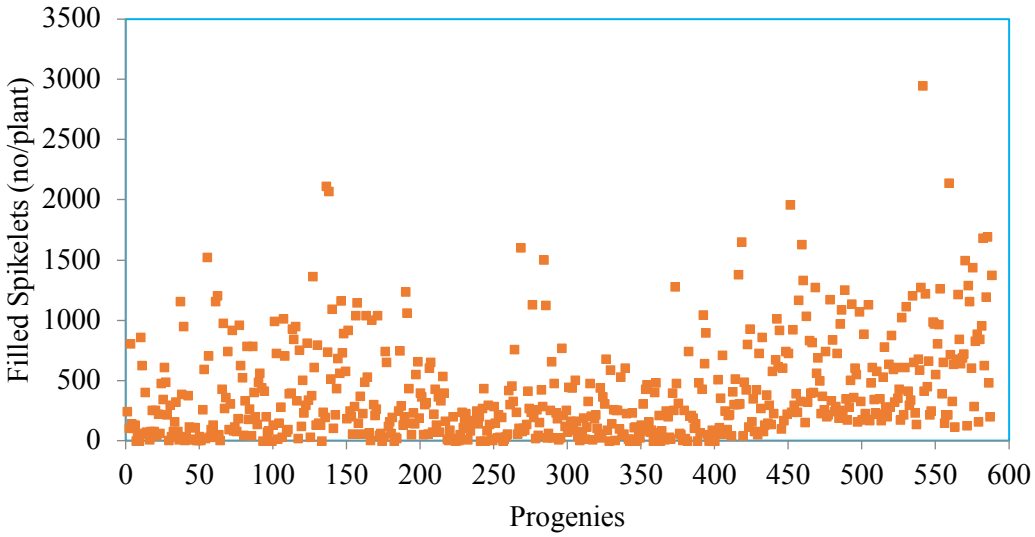
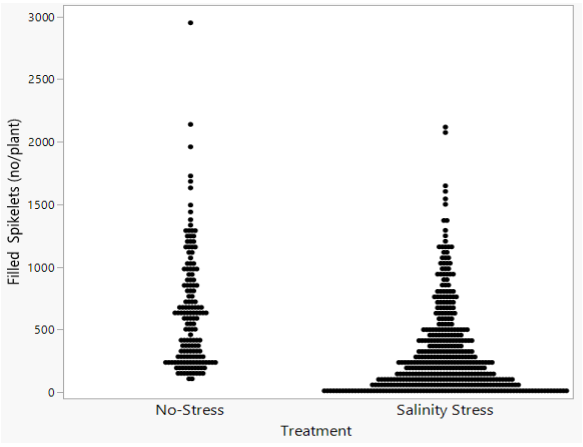


Figure 4.13. Number of filled spikelets per plant of all the BC_1F_2 individuals from the cross, Hasawi x BRRI dhan28.

a. Scatter plot



b. Box plot

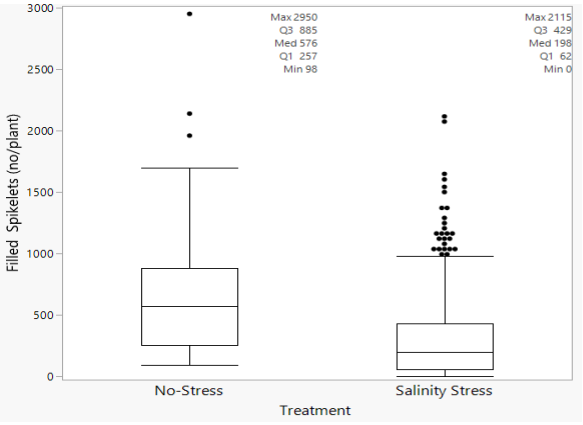


Figure 4.14. Scattered (a) and box (b) plots of the number of filled spikelets per plant of BC_1F_2 individuals under normal and salinity stress at reproductive stage of rice.

The interquartile range of the number of filled spikelets per plant of the progenies of Hasawi x BRRI dhan28 in control treatment was wider than the salinity treatment (Fig. 4.14). The interquartile range for salinity treatments varied from 62 to 429 filled spikelets per plant while it varied from 257 to 885 filled spikelets per plant under no-stress condition. The number of filled spikelets varied significantly between no-stress and salinity treatments (Fig. 4.14, Table 4.2).

Among the plant families of Hasawi x BRRI dhan28, the number of filled spikelets per plant was highly variable (Fig. 4.15). Overall, the control treatment produced more filled spikelets than the salinity treatment. The highest number of filled spikelets was observed in IR131853-10 under control and the individuals of IR131853-6 produced the lowest filled spikelets per plant under salinity stress.

The number of filled spikelets of Hasawi and BRRI dhan28 was 1708 and 2349 per plant under no-stress condition (Table 4.1, Fig. 4.16). The mean number of filled spikelets of the tolerant and sensitive progenies of Hasawi x BRRI dhan28 was 735.7 and 123.6 per plant, respectively; while the progenies produced 637.5 filled spikelets per plant under no-stress condition. Under selective genotyping, it varied significantly between tolerant and sensitive individuals and also between tolerant and control (no-stress) treatments but no variation was found between tolerant and control progenies of this cross (Fig. 4.16, Table 4.2).

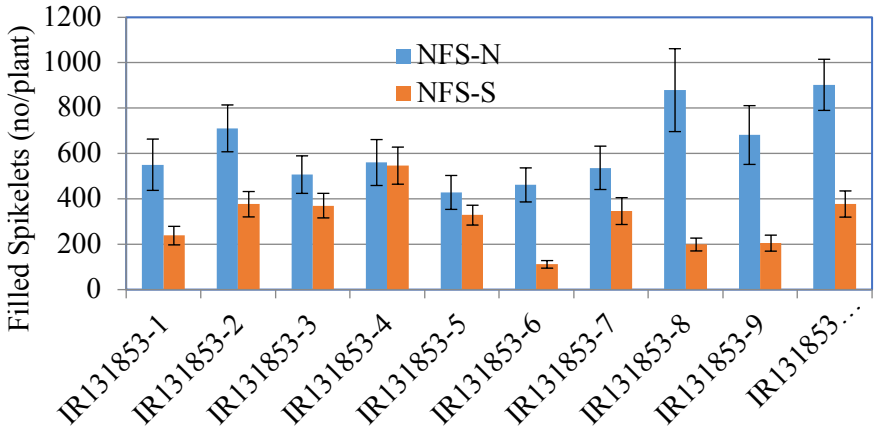


Figure 4.15. Mean number of filled spikelets in the rice families of Hasawi x BRRI dhan28 progenies under control (NFS-N) and salinity stress (NFS-S). Vertical and capped bar indicates standard error of the mean number of filled spikelets of 9 to 46 plants per family.

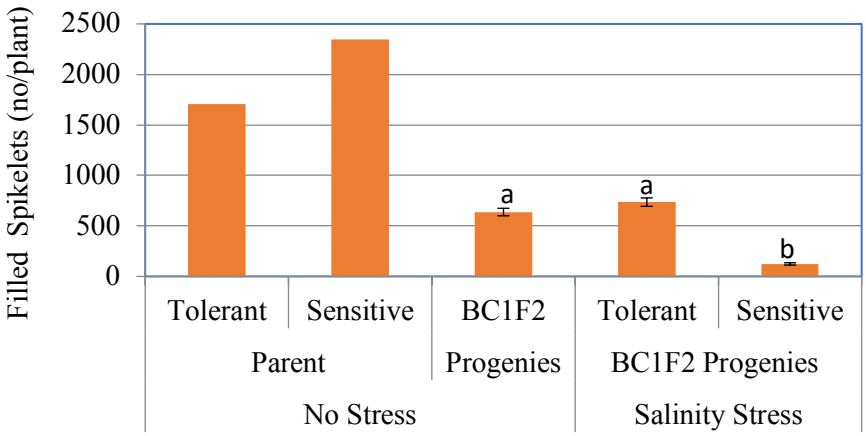


Figure 4.16. Number of filled spikelets per plant of tolerant and sensitive parents under no-stress and their BC_1F_2 progenies under non-stress and salt stress conditions. Vertical and capped bar indicates the mean number of filled spikelets of 153 plants under no-stress, 78 tolerant and 112 sensitive progenies grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

4.4.1.5. Number of Unfilled Spikelets

The number of unfilled spikelets of the BC_1F_2 progenies of Hasawi x BRRI dhan28ranged from 23 to 1602 and 17 to 3316 per plant in control and salinity treatments, respectively (Fig. 4.17, Table 4.1). The number of unfilled spikelets per plant differed significantly ($p<0.001$) between no-stress and salinity treatments (Fig.4.18, Table 4.2).

The interquartile range for the unfilled spikelets per plant of the progenies Hasawi x BRRI dhan28 under no-stress treatment (404 unfilled spikelets per plant) was narrower than the salinity treatment (687 unfilled spikelets per plant) (Fig. 4.18), implies that spikelet sterility was higher under salinity stress condition

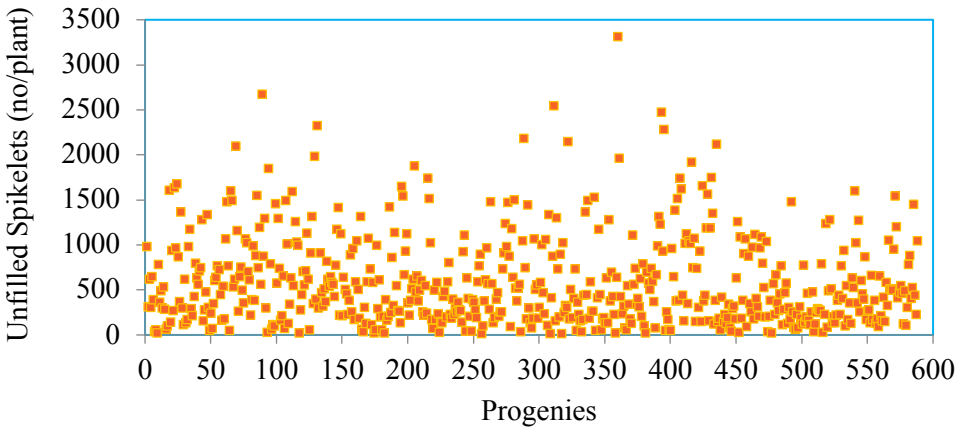
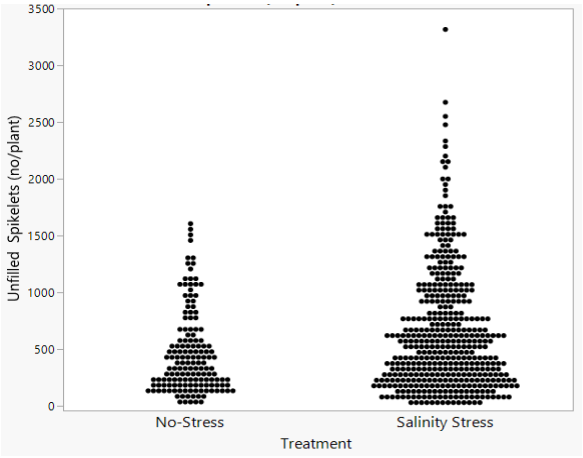


Figure 4.17. Number of unfilled spikelets per plant of all the BC_1F_2 individuals of the cross, Hasawi x BRRI dhan28.

a. Scatter plot



b. Box plot

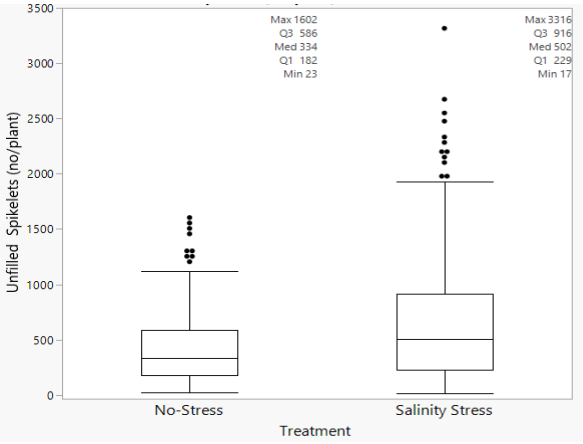


Figure 4.18. Scattered (a) and box (b) plots of the number of unfilled spikelets per plant of BC_1F_2 individuals grown under normal and salinity stress at reproductive stage of rice.

The variability in the number of unfilled spikelets per plant among the families of the cross, Hasawi x BRRI dhan28 is shown in Fig. 4.19. The control treatments had the smaller number of unfilled spikelets than the salinity treatment. Among them, the highest number of unfilled spikelets was observed in IR131853-10 under salinity stress and the lowest in IR131853-5 under no-stress.

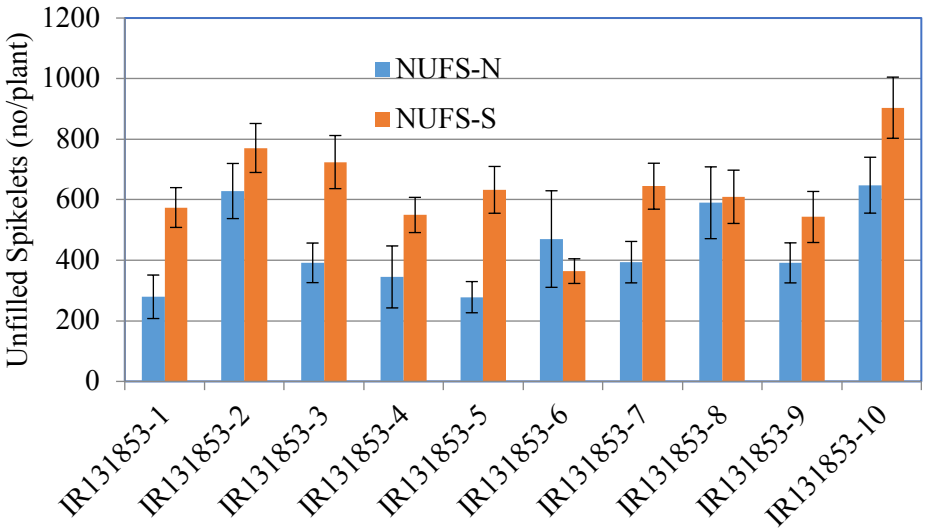


Figure 4.19. Mean number of unfilled spikelets in the rice families of Hasawi x BRRI dhan28 progenies under control (NUFS-N) and salinity stress (NUFS-S). Vertical and capped bar indicates standard error of the mean number of unfilled spikelets of 9 to 46 plants per family.

The number of unfilled spikelets of the tolerant (Hasawi) and sensitive (BRRI dhan28) parents was 436 and 617 per plant, respectively under no-stress condition (Table 4.1, Fig. 4.20). And the number of unfilled spikelets per plant of the tolerant and sensitive progenies was 793.5 and 592.8, respectively under salinity stress. Under no-stress condition, the progenies of Hasawi x BRRI dhan28 produced 448.9 unfilled spikelets per plant. Under selective genotyping, the number of unfilled spikelets per plant differed significantly between the tolerant and sensitive individuals as well as between tolerant and control treatments (Fig. 4.20, Table 4.2).

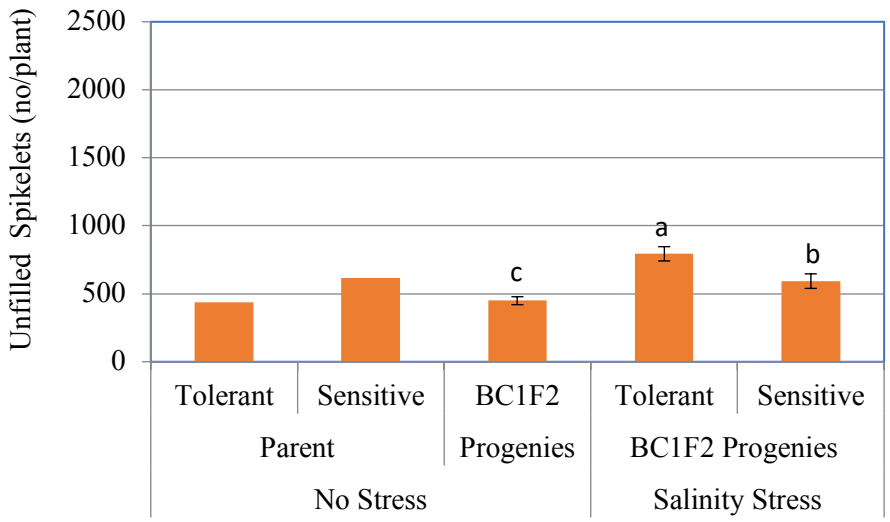


Figure 4.20. Number of unfilled spikelets per plant of tolerant and sensitive parents under no-stress and their BC_1F_2 progenies under no-stress and salt stress condition. Vertical and capped bar indicates standard error of the mean number of unfilled spikelets of 153 progenies under no-stress, 78 tolerant and 112 sensitive progenies under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

4.4.1.6. Percent Filled Spikelets

The percent filled spikelets of the BC_1F_2 progenies of Hasawi x BRRI dhan28ranged from 7.5% to 93.1% under no-stress and 0.0% to 84.7%under salinity stress (Fig. 4.21, Table 4.1). Control and stress treatment were significantly different for the progenies of Hasawi x BRRI dhan28 (Fig. 4.22, Table 4.2).

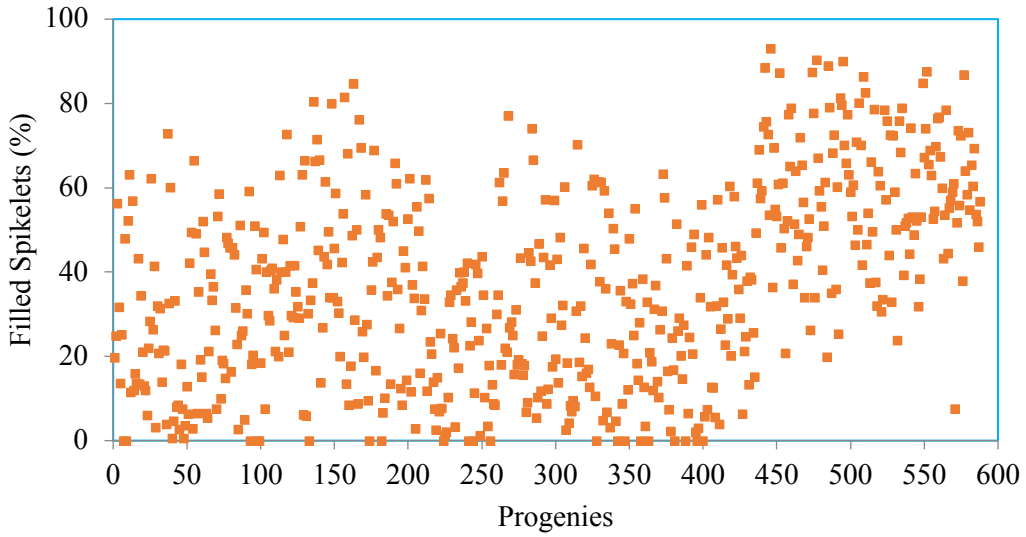
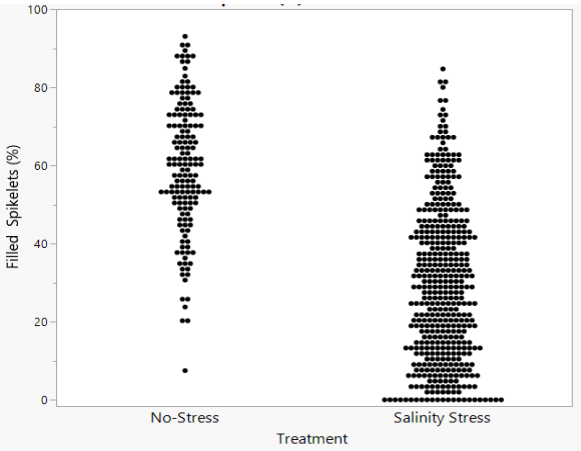


Figure 4.21. Percent filled spikelets per plant of all the BC_1F_2 individuals of the cross, Hasawi x BRRI dhan28.

a. Scatter plot



b. Box plot

Figure 4.22. Scattered (a) and box (b) plots of percent filled spikelets per plant of BC_1F_2 individuals under normal and salinity stress at reproductive stage of rice.

The interquartile range for percent filled spikelets of the BC_1F_2 progenies of Hasawi x BRRI dhan28 was 30.4% under stress condition (Fig. 4.22). The progenies exhibited wider the interquartile range under salinity stress over the no-stress condition (22.9% filled spikelets). The median value of the percent filled spikelets for salinity treatments was 27.6% and it was 59.3% for no-stress condition (Fig. 4.22). This implies that percent filled grains observed under salinity stress significantly reduced over the no-stress treatment (Fig. 4.22, Table 4.2).

The percent filled spikelets varied among the families of Hasawi x BRRI dhan28 (Fig. 4.23). Among them, IR131853-1 and IR131853-4 had higher percent filled spikelets under control condition than other families of Hasawi x BRRI dhan28 and IR131853-6 produced the lowest filled grains under salinity stress.

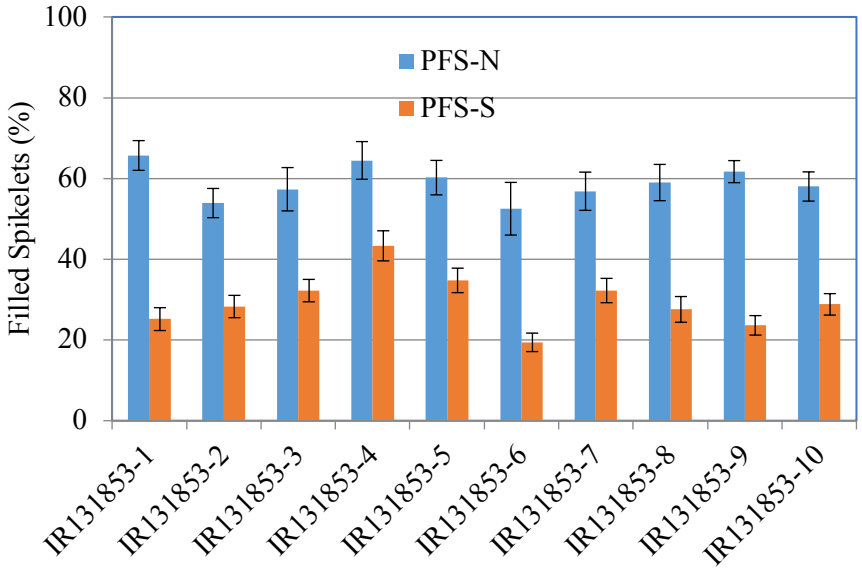


Figure 4.23. Mean percent filled spikelets in the rice families of Hasawi x BRRI dhan28 progenies under control (PFS-N) and salinity stress (PFS-S). Vertical and capped bar indicates standard error of the mean percent filled spikelets of 9 to 46 plants per family.

The percent filled spikelets of the tolerant (Hasawi) and sensitive (BRRI dhan28) parents were similar (79.7% for Hasawi and 79.2% for BRRI dhan28) under no-stress condition (Table 4.1, Fig. 4.24). Under selective genotyping, the percent filled spikelets of the tolerant and sensitive progenies were 49.7% and 20.6%, respectively; and it was 59.1% under no-stress condition (Fig. 4.24). The percent filled spikelets varied significantly

between tolerant and sensitive, and also between control and tolerant progenies of Hasawi x BRRIdhan28 (Fig. 4.24, Table 4.2).

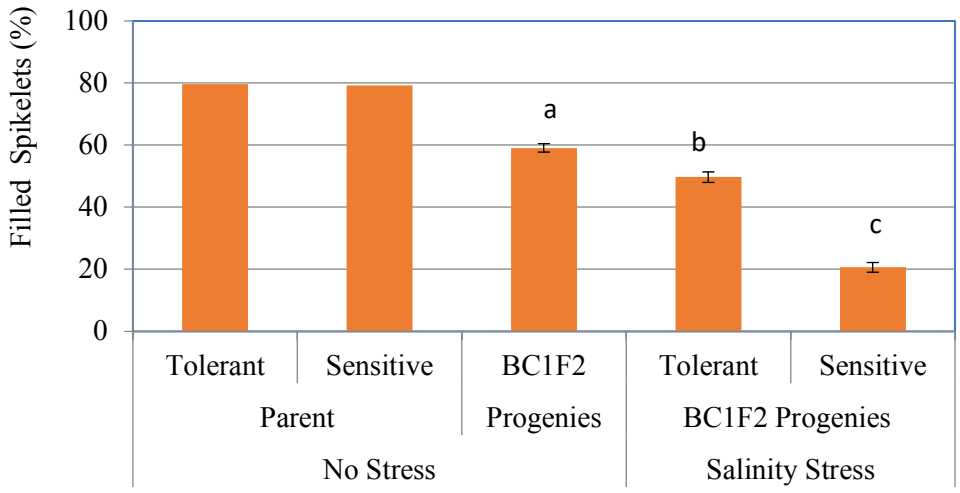


Figure 4.24. Percent filled spikelets per plant of tolerant and sensitive parents under no-stress and their BC_1F_2 progenies under no-stress and salt stress condition. Vertical and capped bar indicates standard error of the mean percent filled spikelets of 153 progenies under no-stress, 78 tolerant and 112 sensitive progenies under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

4.4.1.7. Grain Yield

Grain yield of BC_1F_2progenies from the cross, Hasawi x BRRI dhan28 is shown in Fig. 4.25. Grain yield of the progenies varied from 2.5 g/plant to 80.4 g/plant under control condition and from 0.0 to 43.5 g/plant under salinity stress (Fig. 4.25, Table 4.1). The grain yield of the progenies differed significantly ($p<0.01$) between control and salinity stress treatments (Fig. 4.26, Table 4.2). This indicates existence of genetic variability among the progenies of Hasawi x BRRI dhan28.

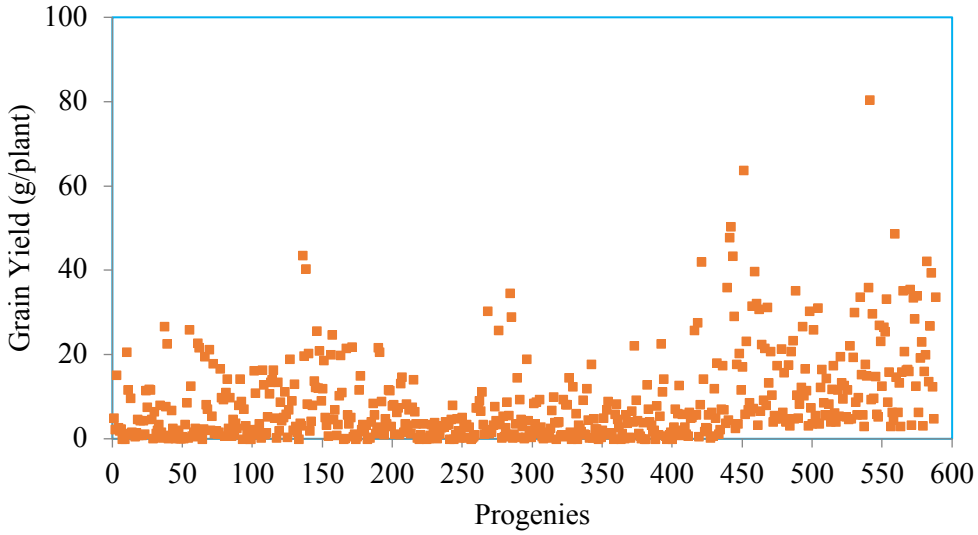
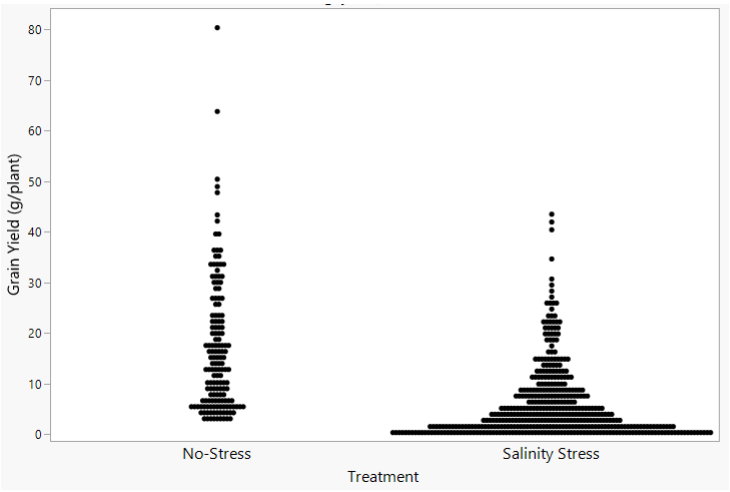


Figure 4.25. Grain yield of all the BC_1F_2 individuals from the cross, Hasawi x BRRI dhan28.

a. Scatter plot



b. Box plot

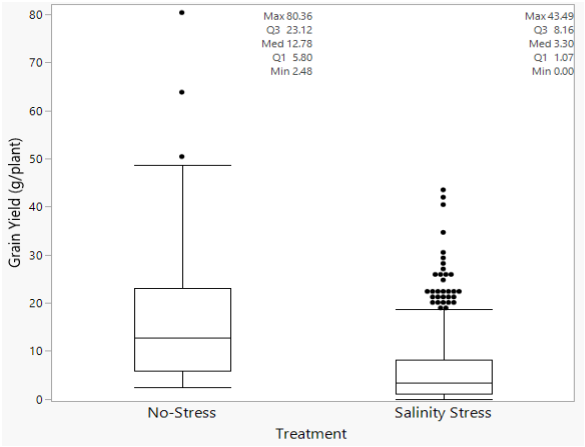


Figure 4.26. Scattered (a) and box (b) plots of grain yield of BC_1F_2 individuals of Hasawi x BRRI dhan28 under normal and salinity stress at reproductive stage of rice.

The interquartile range for the grain yield of the progenies of Hasawi x BRRI dhan28 was 7.1 g/plant for salinity treatment and it was 17.3 g/plant for control (no-stress) treatment (Fig. 4.26) and the median value was 3.30 g/plant and 12.78 g/plant, respectively for stress and no-stress treatment, implies grain yield was significantly reduced under salinity stress condition.

The grain yield of the progenies of Hasawi x BRRI dhan28 varied among the plant families (Fig. 4.27). Higher grain yield was observed among the control plant families grown under non-stress condition over those grown under salinity stress. The family, IR131853-1 produced the highest yield under control and IR131853-6 produced the lowest grain yield under salinity stress. This indicates genetic variability among the plant families of this cross.

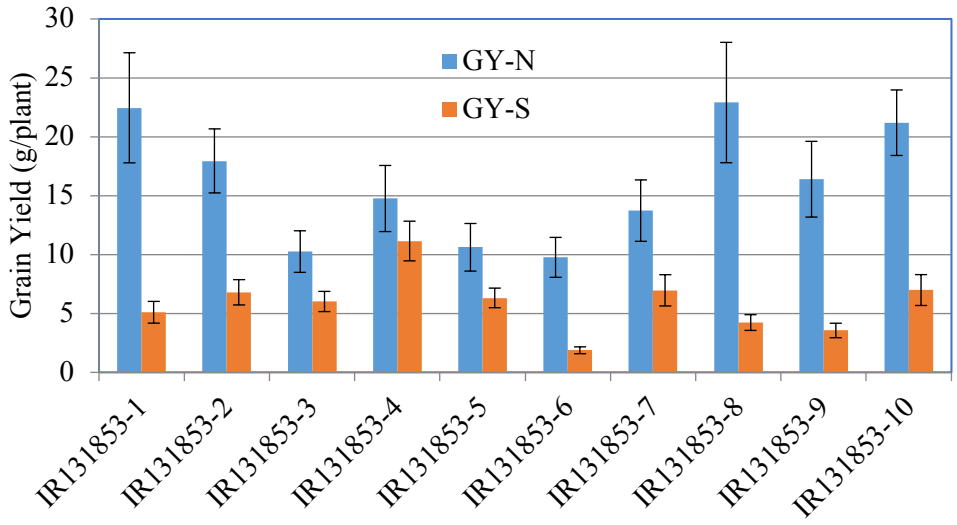


Figure 4.27. Mean grain yield in the rice families of Hasawi x BRRI dhan28 progenies under control (GY-N) and salinity stress (GY-S). Vertical and capped bar indicates standard error of the mean grain yield of 9 to 46 plants per family.

The grain yield of tolerant (Hasawi) and sensitive (BRRI dhan28) parents under normal growing condition (no-stress) was 48 g/plant and 55 g/plant, respectively (Fig. 4.28, Table 4.1). Under selective genotyping, mean grain yield of the tolerant and sensitive progenies of Hasawi x BRRI dhan28 was 15.1 g/plant and 2.3 g/plant, respectively (Fig. 4.28). And it was 16.5 g/plant under no-stress treatment. The grain yield of the tolerant

progenies of Hasawi x BRRI dhan28 did not significantly vary with the no-stress treatment; but highly significant difference was observed between the grain yield of sensitive progenies with the tolerant progenies grown under salinity stress and the progenies grown under control condition (Table 4.2). Overall, the yield reduction of the tolerant and sensitive progenies over those grown under control condition was 8.6% and 87.1%, respectively (Table 4.1, Fig. 4.28).

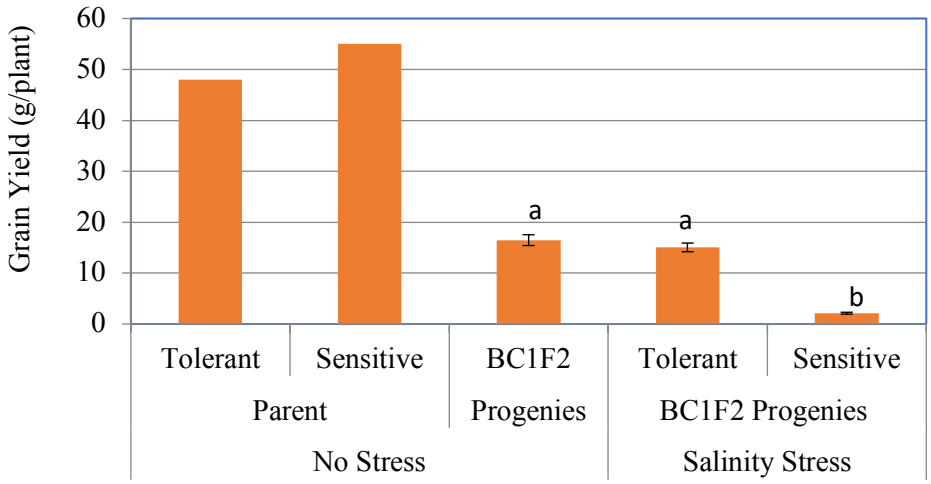


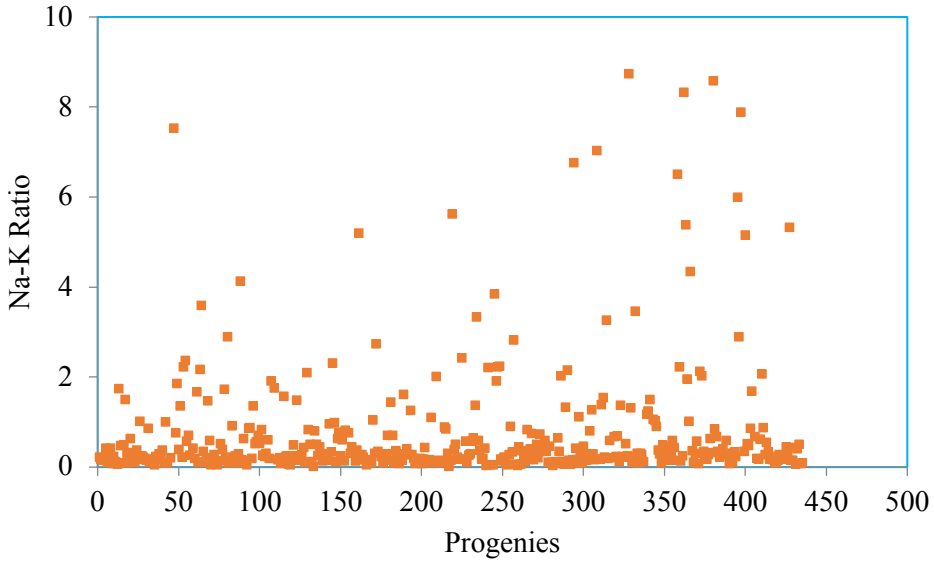
Figure 4.28. Grain yield of tolerant and sensitive parents under no-stress and their BC_1F_2 progenies of Hasawi x BRRI dhan28 under no-stress and salinity salt stress condition. Vertical and capped bar indicates standard error of the mean grain yield of 153 progenies under no-stress, 78 tolerant and 112 sensitive progenies grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

4.4.1.8. Sodium-Potassium (Na^+-K^+) Ratio

The Na^+ and K^+ concentration in the flag leaf of the BC_1F_2 progenies of Hasawi x BRRI dhan28 varied from 0.025 to 6.76 mmol/g and 0.225 to 1.56 mmol/g, respectively (Fig. 4.29). The concentration of K^+ was higher than that of Na^+. The Na^+-K^+ ratio of the BC_1F_2 progenies of Hasawi x BRRI dhan28 ranged from 0.03 to 8.7under salinity stress of EC 10 dS/m at the reproductive stage (Fig. 4.29).

The mean Na^+-K^+ ratio of the plant families of BC_1F_2 progenies of Hasawi x BRRI dhan28 ranged 0.3 to 1.2 (Fig. 4.30). The plant family, IR131853-1 had the lowest and IR131853-9 exhibited the highest Na^+-K^+ ratio. Overall, Na^+-K^+ ratio significantly varied between tolerant and sensitive progenies of Hasawi x BRRI dhan28 (Fig. 4.30, Table 4.2).

a. Scattered Plot



b. Box Plot

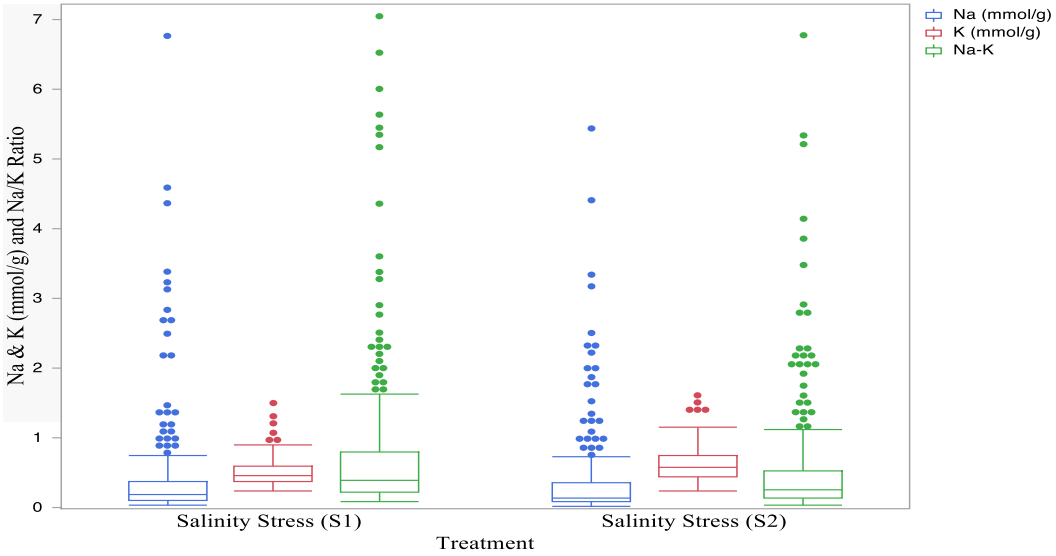
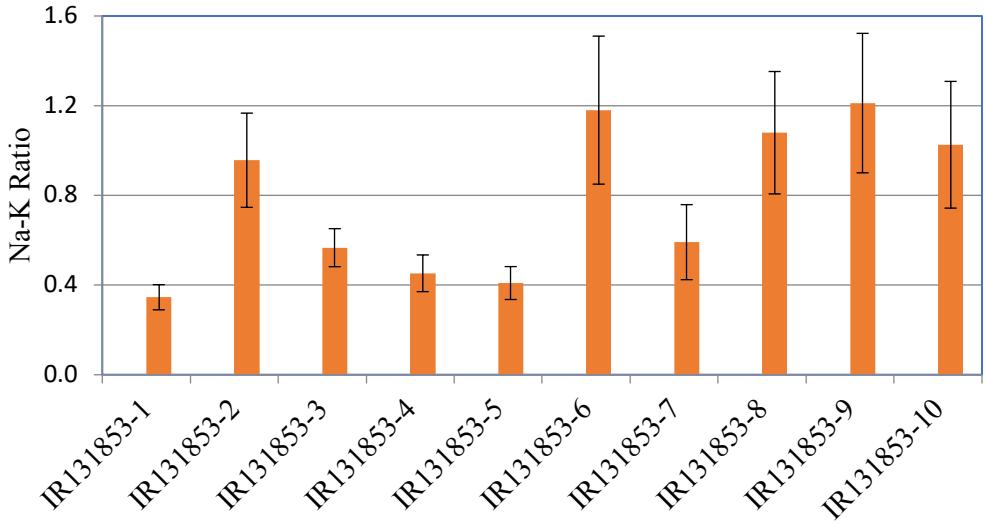


Figure 4.29. Scattered (a) and box (b) plots of Na-K ratio of BC_1F_2 progenies under salinity stress of the cross, Hasawi x BRRI dhan28.

a. Na-K Ratio of Plant Family



b. Na-K Ratio of Selected Progenies

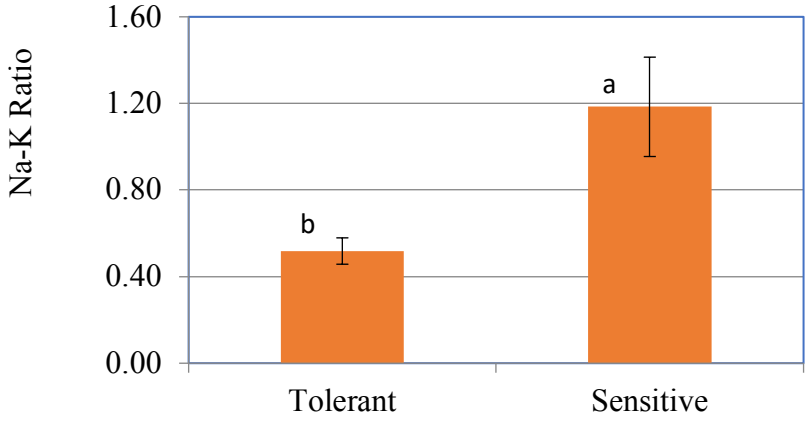


Figure 4.30. Mean Na-K ratio of the rice families (a), tolerant and sensitive (b) progenies of Hasawi x BRRI dhan28 under salinity stress. Vertical and capped bar indicates standard error of the mean Na-K ratio of 42 to 46 plants per family, and 78 tolerant and 112 sensitive plants grown under salinity stress. Values with the same letter are not significantly different at 5% level of significance.

4.4.2. Correlation Analysis of Yield and Agronomic Components

Correlation coefficient analysis measures the association between two traits. When establishing the relationship between salt-stress and overall phenotypic performance, correlation analysis is useful (Gomez and Gomez, 1984; Sokal and Rohlf, 1995; Steel *et al.* 1997). It provides an indication of the degree of association between two variables that are considered to be independent. However, association detected from correlation coefficients may not necessarily be attributed to a single variable but rather to the number of interdependent variables.

Highly significant positive correlations between grain yield and number of filled spikelets (r=0.93), percent filled spikelet (r=0.68), and productive tillers (r=0.54) were observed for 435 BC_1F_2 mapping population from the cross, Hasawix BRRI dhan28 (Fig. 4.31). The percent unfilled spikelets (r = -0.68) and SES score (r = -0.80) also showed significantly negative correlation with the grain yield.

The number of filled spikelets per plant had the highest positive correlation and the percent unfilled spikelets showed significantly negative correlation with the grain yield (Fig. 4.31). The number of unfilled spikelets per plant showed positive but weak correlation with grain yield of the progenies of Hasawi x BRRI dhan28, which is unexpected. Mondal et al. (2019) observed similar positive correlation with grain yield and number of unfilled spikelets of the F_2 mapping population from the crosses between NSIC Rc222 (sensitive parent) and BRRI dhan47 (tolerant parent). The possible reason may be the tendency of producing excess sterile spikelets by the tolerant parents under salt stressed environment.

Salinity evaluation score (SES) showed negative and highly significant correlation with plant height, productive tiller per plant, panicle length, number of filled spikelets per plant, percent filled spikelets and grain yield (Fig. 4.31). And it showed highly significant positive correlation with percent unfilled spikelets. Therefore, SES score might be an initial stress indicator to identify salt-tolerant and salt-sensitive genotypes. In addition to SES score, grain yield, filled spikelets and productive tillers are considered the ultimate stress indicators for salinity tolerance in rice.

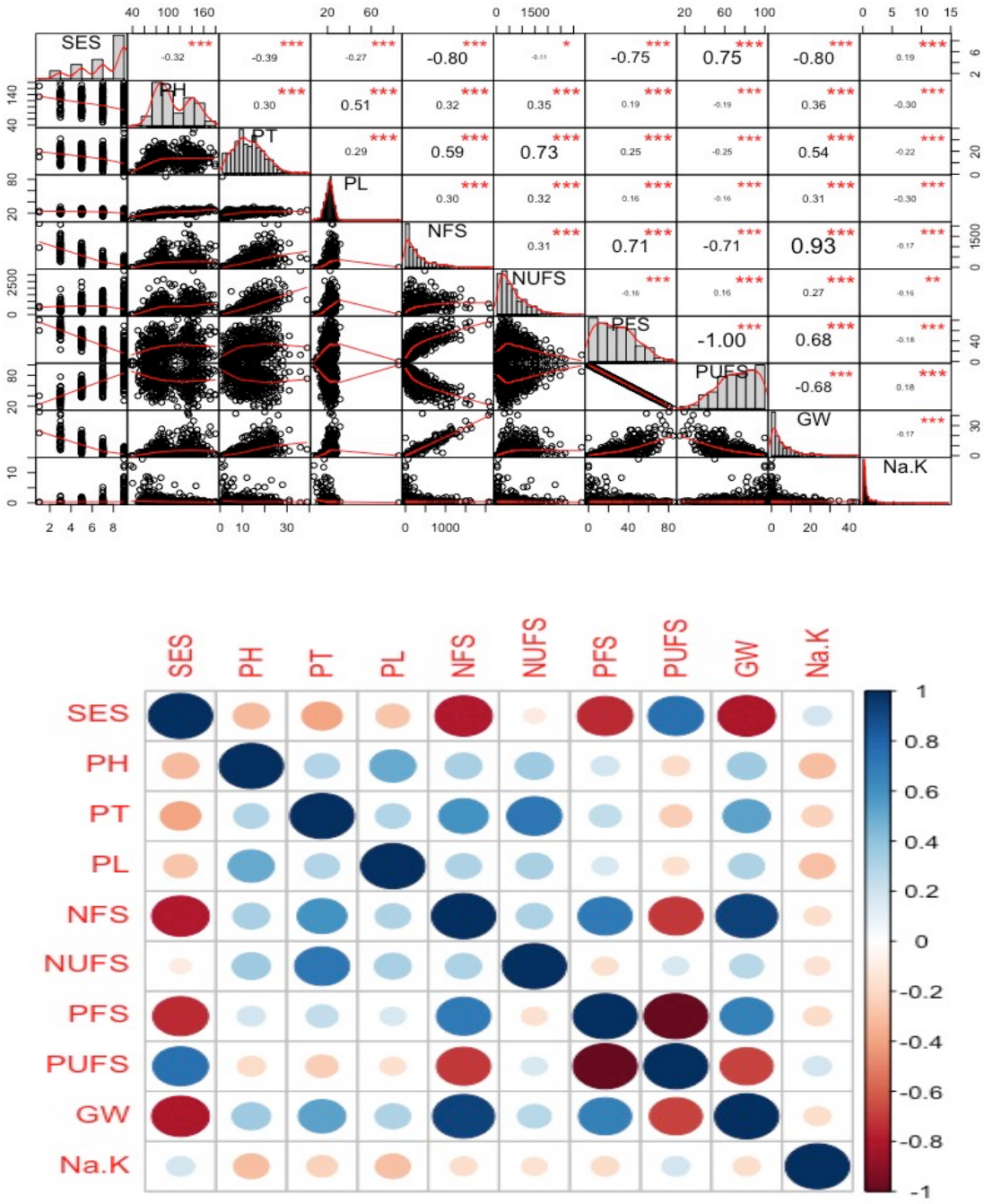


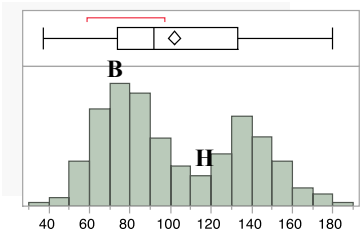
Figure 4.31. Phenotypic distribution and correlation coefficients for grain yield and agronomic components of BC_1F_2 individuals from Hasawi x BRRI dhan28. Pearson correlation coefficient (top) and correlogram (bottom) among the traits under salinity stress of 10 dS/m at the reproductive stage of rice plant.

4.4.3. Frequency Distribution

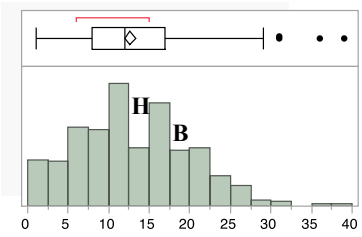
Out of the 435 BC_1F_2 progenies of Hasawi x BRRI dhan28, 124 plants were tolerant and 311 plants were sensitive. Among them, 78 plants were selected as highly tolerant and 112 plants were selected as highly sensitive based on selective genotyping. They represent two extreme tails of the phenotypic distribution based on their responses to salt stress for the traits, those described in the subsequent sections. Frequency distributions are shown in Fig. 4.32.

4.4.3.1. Plant Height

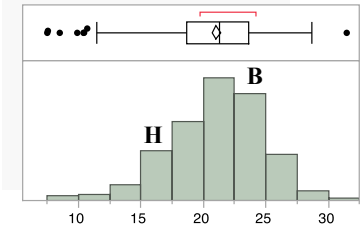
The segregation pattern for the plant height ofBC_1F_2 population of Hasawi x BRRI dhan28 is shown in Fig. 4.32. Some BC_1F_2 progenies had higher plant height than the tolerant parent and some had lower than the sensitive parent under salinity stress. The plant height of Hasawi and BRRI dhan28 was 124.0 cm and 73.0 cm, respectively (Table 4.1, Fig. 4.32). The mean plant height the progenies was 102.0 cm that varied from 37.0 cm to 180.0 cm. The lower and upper 95th percentile of plant height was 98.9 cm and 105.2 cm, respectively with a median of 92.0 cm. The plant height showed positive skewness (+0.36) (Table 4.1). Most of the plant height (95.2%) ranged from 50 cm to 160 cm. Out of 435 BC_1F_2 progenies, the plant height of 144 individuals (33.1%) were taller than the tolerant parent, Hasawi and 104 individuals (23.9%) had shorter plants than the sensitive parent, BRRI dhan28. Overall, 43% (187 individuals) of the total population had plant height in between the tolerant and sensitive plants.



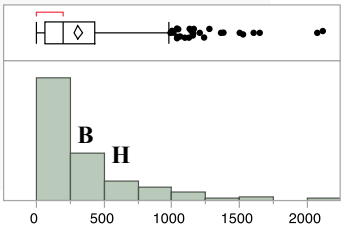
a. Plant Height (cm)



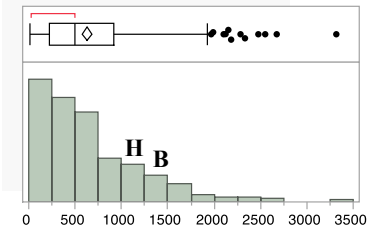
b. Productive Tiller (no/plant)



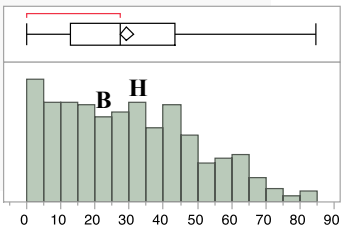
c. Panicle length (cm)



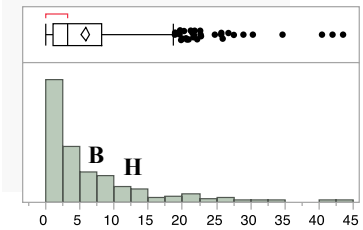
d. Filled Spikelets (no/plant)



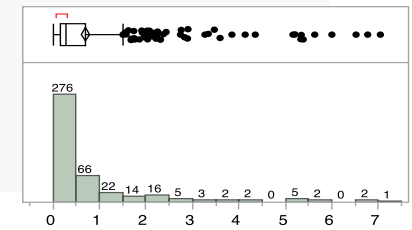
e. Unfilled Spikelets (no/plant)



f. Filled Spikelets (%)



g. Grain Yield (g/plant)



h. Na-K Ratio

Figure 4.32. Frequency distribution of the progenies of Hasawi x BRRI dhan28 grown under salinity stress of EC 10 dS/m at the reproductive stage of rice plant.

4.4.3.2. Productive Tillers

The frequency distribution of number of productive tillers per hill of the BC_1F_2 population from the cross, Hasawi x BRRI dhan28 are shown in Fig. 4.32. Some BC_1F_2 individuals had higher productive tillers than the tolerant parent and some had lower than the sensitive parent under saline stress (Figs. 4.6-4.8).

The number of productive tillers of the tolerant and sensitive parents was 13 and 19 per plant, respectively under stress condition (Figs. 4.6-4.8). The number of productive tillers per plant of the BC_1F_2 population of Hasawi x BRRI dhan28 ranged from 1 to 39 which follow the positively skewed (+0.44) distribution (Table 4.1). Out of 435 BC_1F_2 progenies, 188 individuals (43.2%) had more productive tillers than the tolerant parent, while only 74 individuals (17.0%) had more productive tillers than the sensitive parent (Fig. 4.32). Overall, 30.8% of the total population had productive tillers in between tolerant and sensitive parents (8-19 productive tillers/plant).

4.4.3.3. Panicle Length

The panicle length of Hasawi and BRRI dhan28 was 15.5 cm and 24.5 cm, respectively (Figs. 4.10-4.12, 4.32). The mean panicle length of the total population was 21.0 cm and that ranged from 7.5 to 31.5 cm. The panicle length of the lower and upper 95^{th} percentile was 20.7 and 21.4 cm, respectively (Fig. 4.10) and the distribution was negatively skewed (-0.53) (Table 4.1). Out of 435 BC_1F_2 progenies, 400 (91.9%) individuals had the panicle length ranging from 15.0 to 27.5 cm. Among them, 401 individuals (92.2%) had higher panicle length than the tolerant parent, while only 71 individuals (16.3%) had higher

panicle length than the sensitive parent. Overall, the panicle length of 331 individuals (76.1%) was in between that of the tolerant and sensitive parents.

4.4.3.4. Number of Filled Spikelets

The number of filled spikelets of the tolerant (Hasawi) and sensitive (BRRI dhan28) parents was 550 and 356 per plant, respectively (Figs. 4.14-4.16, 4.32). The mean of the whole population for this trait was 307.6 with a median value of 198.0 under salinity stress condition. The range of whole BC_1F_2 population for the number of filled spikelets per plants varied from 0 to 2115 (Table 4.1). The upper 95th percentile and the lower 95th percentile of number of filled spikelets was 340.1 and 275.1, respectively (Fig. 4.14) and the distribution is positively skewed (+1.89). Out of 435 BC_1F_2 progenies for Hasawi x BRRI dhan28, 348 (80%) individuals had 0 to 500 filled spikelets per plant. Among them, 78 (17.9%) individuals had more number of filled spikelets per plant than the tolerant parent (550), and 300 individuals (69%) had less number of filled spikelets per plant than the sensitive parent (356). Overall, only 13.1% (57 individuals) of the total population had the number of filled spikelets in between the tolerant and sensitive parents.

4.4.3.5. Number of Unfilled Spikelets

The number of unfilled spikelets of the tolerant (Hasawi) and sensitive (BRRI dhan28) parents was 1230 and 1377 per plant, respectively (Fig. 4.32). The mean of the whole population for this trait was 633.4 and that varies from 17 to 3316 unfilled spikelets per plant with median of 502 (Table 4.1, Figs. 4.18-4.20). The lower and upper 95th percentile of number of unfilled spikelets was 583.5 and 683.3, respectively and the

distribution is positively skewed (+1.40). Out of 435 BC_1F_2 progenies, 301 (69.2%) individuals had 0 to 750 unfilled spikelets per plant. Among them, 375 (86.2%) individuals from Hasawi x BRRI dhan28 had a lesser number of unfilled spikelets per plant than the tolerant parent (1230), and only 44 individuals (10.1%) had a higher number of unfilled spikelets per plant than the sensitive parent (1377). Overall, only 3.7% (16 individuals) of the progenies of this cross produced unfilled spikelets in between two parents.

4.4.3.6. Percent Filled Spikelets

The percentage of filled spikelets of the tolerant (Hasawi) and sensitive (BRRI dhan28) parents was 30.9% and 20.56%, respectively (Fig. 4.32). The mean of the whole BC_1F_2 population under salinity stress for this trait was 29.4% and that varies from 0 to 84.7% (Table 4.1, Figs. 4.22-4.24). The lower and upper 95th percentile was 27.5% and 31.2%, respectively with a median value of 27.6%. The distribution of this population is positively skewed (+0.42). Out of 435 BC_1F_2 progenies, 364 (83.7%) individuals had percent filled spikelets ranged from 0 to 50%. Among them, 197 (45.3%) individuals had a greater percentage of filled spikelets than the tolerant parent (30.9%) and 167 individuals (38.4%) had a smaller percentage of filled spikelets than the sensitive parent (20.6%). Overall, 16.3% (71 BC_1F_2 individuals) of the individuals had the filled spikelets in between tolerant and sensitive parents.

4.4.3.7. Grain Yield

The grain yield of the tolerant (Hasawi) and sensitive (BRRI dhan28) parents was 14.04 g/plant and 6.45 g/plant, respectively (Fig. 4.32). The mean of the whole population for the Hasawi x BRRI dhan28 was 5.85 g/plant and that varies from 0 to 43.5 g/plant (Table 4.1, Figs. 4.30-4.33). The lower and upper 95th percentile of grain yield was 5.19 and 6.52 g/plant, respectively with a median value of 3.3 g/plant. The distribution of this population is positively skewed (+2.11) (Fig. 4.37). Out of 435 BC_1F_2 progenies, 343 (78.9%) individuals had grain yield ranged from 0 to 10 g/plant. Among them, 51 (11.7%) individuals showed higher grain yield than the tolerant parent (14.04g/plant) and 297 individuals (68.3%) had lower grain yield than the sensitive parent (6.45 g/plant). Like the progenies from CSR28 x BRRIdhan28, 20% this cross produced grain yield in between tolerant and sensitive parents.

4.4.3.8. Sodium-Potassium (Na^+-K^+) Ratio

The Na^+-K^+ ratio of the tolerant (Hasawi) and sensitive (BRRI dhan28) parents was 1.927 and 3.105 respectively (Fig. 4.32, Figs. 4.34-4.35). The mean of the whole BC_1F_2 population for Na^+-K^+ was 0.865 and that varies from 0.002 to 7.041 (Table 4.1). The upper and lower 95th percentile of the Na^+-K^+ ratio was 1.026 and 0.703, respectively with a median value of 0.3. The distribution of this population is positively skewed (+4.458) (Fig. 4.37, Table 4.1). Out of 418 BC_1F_2 progenies, the Na^+-K^+ ratio of 342 (82.2%) individuals ranged from 0 to 1.

4.4.4. QTL Mapping for Salinity Tolerance at Reproductive Stage

QTLs related with yield and yield components and some important agronomic components under salinity stress were identified through Inclusive composite interval mapping (CIM) using IciMapping. Table 4.3 represents the name of the QTLs identified, chromosomal location, nearest marker interval, peak LOD, phenotypic variation explained by QTL (R^2) and direction of the phenotypic effect (additive effect) and allelic effect. A total of 35 QTLs were identified in seven traits: plant height (PH), productive tillers (PT), panicle length (PL), number of filled spikelets (NFS), number of unfilled spikelets (NUFS), percent filled spikelet (PFS), percent unfilled spikelets (PUFS) and grain yield (GY). All these QTLs were detected in all chromosomes, except in chromosome 5 and chromosome 10 (Table 4.3). The phenotypic variation of the identified QTLs individually accounted for 3.86% to 22.69%.

Table 4.3. QTLs identified using Inclusive Composite Interval Mapping (IciMapping) for agronomic components of the BC_1F_2 progenies from Hasawi x BRRI dhan28 under salt stress of EC 10 dS/m at the reproductive stage of rice.

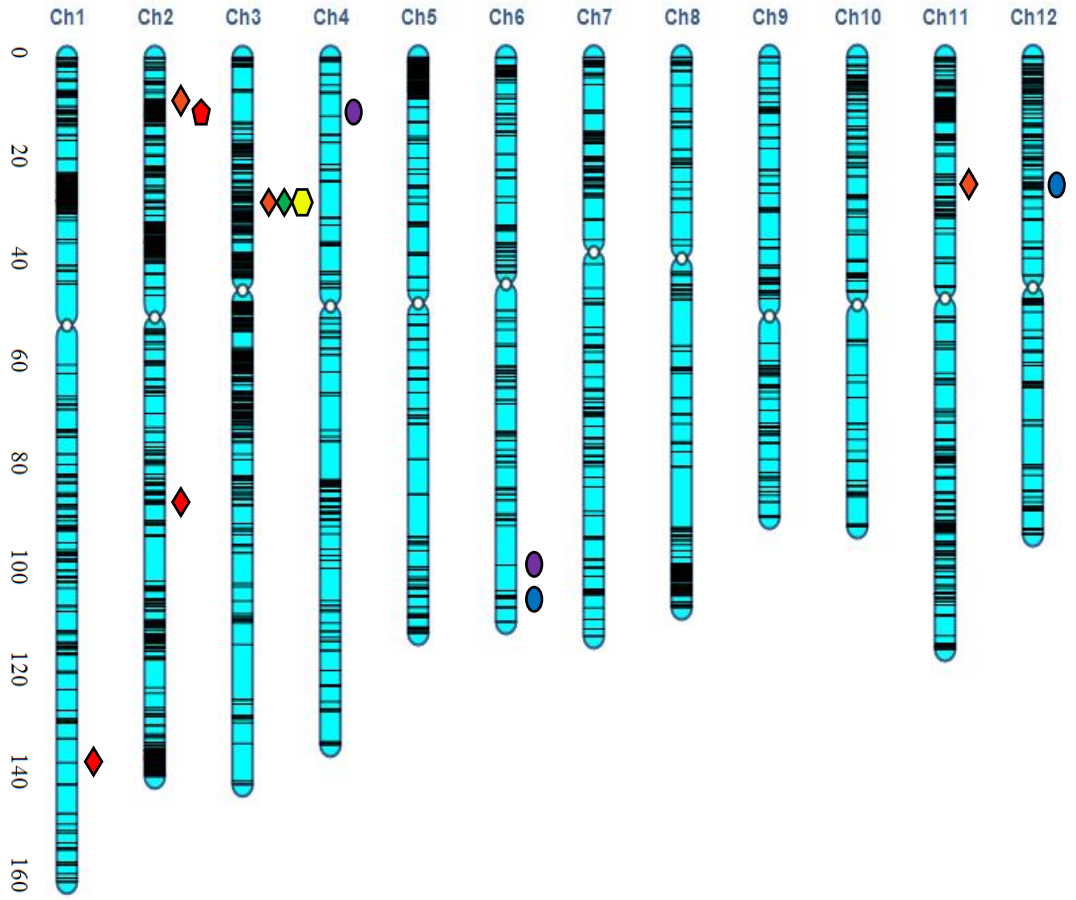
Trait Name	Chromo-some	QTL	Position	Left Marker	Right Marker	LOD	PVE (%)	Additive Effect	Allelic Effect
Plant Height	1	*qPH1.1*	138.67	CM020682.1_33842918_1	CM020682.1_34965920_1	14.87	27.03	-22.46	Hasawi
	2	*qPH2.1*	91.23	CM020683.1_22803914_2	CM020683.1_23139808_2	7.47	9.55	-12.36	Hasawi
	4	*qPH4.1*	34.36	CM020685.1_6844517_4	CM020685.1_8787440_4	3.09	3.87	-9	Hasawi
Productive Tiller	2	*qPT2.1*	7.23	CM020683.1_1744356_2	CM020683.1_2059119_2	7.02	11.19	-0.01	Hasawi
	3	*qPT3.1*	27.08	CM020684.1_6731611_3	CM020684.1_6810365_3	6.12	11.92	-0.34	Hasawi
	6	*qPT6.1*	118.92	CM020687.1_29137956_6	CM020687.1_29925158_6	4.77	11.65	3.79	BRRI dhan28
	8	*qPT8.1*	31.22	CM020689.1_7802407_8	CM020689.1_8320104_8	4.19	5.57	4.12	BRRI dhan28
	11	*qPT11.1*	26.37	CM020692.1_6481151_11	CM020692.1_6638823_11	7.21	13.41	3.23	BRRI dhan28
	11	*qPT11.2*	20.37	CM020692.1_4890980_11	CM020692.1_5380140_11	3.64	8.57	0.37	BRRI dhan28
Panicle Length	2	*qPL2.1*	91.23	CM020683.1_22803914_2	CM020683.1_23139808_2	5.24	6.58	-1.16	Hasawi
	3	*qPL3.1*	32.08	CM020684.1_7851800_3	CM020684.1_8057740_3	3.02	4.25	0.17	BRRI dhan28
	6	*qPL6.1*	118.92	CM020687.1_29137956_6	CM020687.1_29925158_6	6.37	14.25	2	BRRI dhan28
	6	*qPL6.2*	102.92	CM020687.1_24647518_6	CM020687.1_26815006_6	3.19	8.31	1.32	BRRI dhan28
	6	*qPL6.3*	93.92	CM020687.1_23254259_6	CM020687.1_23991427_6	3.79	6.39	0.78	BRRI dhan28
	6	*qPL6.4*	111.92	CM020687.1_26815088_6	CM020687.1_28217292_6	3.14	3.9	0.75	BRRI dhan28
	12	*qPL12.1*	26.22	CM020693.1_6528664_12	CM020693.1_6924636_12	8.65	11.91	-0.44	Hasawi

Table 4.3. Continued

Trait Name	Chromo-some	QTL	Position	Left Marker	Right Marker	LOD	PVE (%)	Additive Effect	Allelic Effect
No of Filled Spikelets	3	*qNFS3.1*	27.08	CM020684.1_6731611_3	CM020684.1_6810365_3	6.56	17.94	-6.26	Hasawi
	3	*qNFS3.2*	87.08	CM020684.1_21729110_3	CM020684.1_21917452_3	3.89	8.22	-241.51	Hasawi
	6	*qNFS6.1*	97.92	CM020687.1_24336222_6	CM020687.1_24647518_6	3.61	11.51	12.88	BRRI dhan28
	7	*qNFS7.1*	52.79	CM020688.1_12463596_7	CM020688.1_13718255_7	3.48	17.39	-129.05	Hasawi
	8	*qNFS8.1*	4.22	CM020689.1_949460_8	CM020689.1_1275124_8	3.82	16.57	63.01	BRRI dhan28
	11	*qNFS11.1*	76.37	CM020692.1_18857719_11	CM020692.1_19789359_11	3.96	12.34	-509.21	Hasawi
	12	*qNFS12.1*	82.22	CM020693.1_19382691_12	CM020693.1_21580067_12	3.03	15.03	-435.44	Hasawi
No UFS	2	*qNUFS2.1*	10.23	CM020683.1_2221957_2	CM020683.1_2585844_2	5.25	11.42	-229.78	Hasawi
Percent Filled Spikelets	4	*qPFS4.1*	9.36	CM020685.1_2019282_4	CM020685.1_3262402_4	4.27	14.29	11.78	BRRI dhan28
	4	*qPFS4.2*	99.36	CM020685.1_24786787_4	CM020685.1_25615450_4	3.55	6.51	4.6	BRRI dhan28
	6	*qPFS6.1*	111.92	CM020687.1_26815088_6	CM020687.1_28217292_6	7.81	14.91	7.55	BRRI dhan28
Grain Yield	1	*qGY1.1*	123.67	CM020682.1_30842423_1	CM020682.1_31267467_1	6.18	7.81	-0.44	Hasawi
	3	*qGY3.1*	27.08	CM020684.1_6731611_3	CM020684.1_6810365_3	7.31	11.56	-0.1	Hasawi
	3	*qGY3.2*	87.08	CM020684.1_21729110_3	CM020684.1_21917452_3	4.45	5.79	-4.33	Hasawi
	3	*qGY3.3*	2.08	CM020684.1_510306_3	CM020684.1_528354_3	3.58	4.34	2.62	BRRI dhan28
	4	*qGY4.1*	98.36	CM020685.1_24368860_4	CM020685.1_24786787_4	4.27	10.04	-1.16	Hasawi
	6	*qGY6.1*	42.92	CM020687.1_10644021_6	CM020687.1_10866598_6	7.07	19.24	0.32	BRRI dhan28
	6	*qGY6.2*	106.92	CM020687.1_24647518_6	CM020687.1_26815006_6	4.51	4.97	2.09	BRRI dhan28
	9	*qGY9.1*	75.12	CM020690.1_18345383_9	CM020690.1_19048905_9	4.12	4.74	2.2	BRRI dhan28

4.4.4.1. Linkage Analysis

Linkage maps of 12 chromosomes were created based on genotypic data of 190 BC1F2 individuals from cross between Hasawi and BRRI dhan28, with 6209 polymorphic SNP markers using IciMapping software. (Fig. 4.33). Initially, millions of markers per chromosome were filtered using TASSEL (Traits Analysis by Association, Evolution and Linkage) software. Firstly, VCF file for each chromosome was uploaded into TASSEL and after uploading following criteria was selected for filtering the unwanted markers: minimum count was 192; minimum allele frequency was 0.05 and maximum allele frequency was 1.0, minimum and maximum heterozygous proportion was 0.05 and 1.0, respectively. The percentage of total phenotypic variation explained by QTL identified for each trait was estimated as R^2 value. The data were permuted 1000 times to confirm the presence of each QTL across the 12 chromosomes. Total length of the distribution of the 6209 markers was 1556.57 cM or 389.14 Mb.



Symbol	Traits
◆	Plant Height
◆	Productive Tillers
●	Panicle Length

Symbol	Traits
◆	No Filled Spikelets
⬟	No Unfilled Spikelets
●	% Filled Spikelets
⬢	Grain Yield

Figure 4.33. Genetic linkage map of 12 chromosomes based on the BC_1F_2 mapping population of Hasawi x BRRI dhan28 under salinity stress of EC 10 dS/m at reproductive stage of rice. Significant QTLs are shown at the right side of each chromosome based on the physical position (cM) of the SNP markers. Ch represents chromosomes and cM represents position in centimorgan.

4.4.4.2. Plant Height

Three QTLs were identified for the plant height on the chromosomes 1, 2 and 4 (Table 4.3; Figs. 4.33 and 4.34) under salinity stress at the reproductive stage of rice. Among them, two QTLs on chromosome 1 (*qPH1.1*) and chromosome 2 (*qPH2.1*) were significant ($p<0.05$). They were mapped on the long arm of the chromosome 1 and 2 at 139.67 cM, and 91.23 cM, respectively. All the significant QTLs were contributed by salt tolerant parent, Hasawi. The largest effect was found in *qPH1.1* with a LOD value of 14.87 that can explain 27.03% phenotypic variation followed by *qPH2.1* with the LOD of 7.47 that can explain 9.55% phenotypic variations. In all cases, the additive effect was negative.

4.4.4.3. Productive Tillers

Six QTLs were identified for the productive tillers per plant on the chromosomes 2, 3, 6, 8 and 11 (Table 4.3, Figs. 4.33 and 4.35). Among them, three QTLs (*qPT2.1, qPT3.1, qPT11.1*) were reported as significant ($p<0.05$). They all were mapped on the short arm of chromosome 2, 3 and 11 at 7.23 cM, 27.08 cM, and 26.37cM, respectively. The other QTLs were identified on the long arm of chromosome 6 and short arm of chromosome 8 and 11 with a LOD value of 4.78, 4.18 and 3.64, respectively. Of the significant QTLs, the largest effect was found in *qPT11.1* with a LOD value of 7.20 that can explain 13.41% phenotypic variation. The additive effect of *qPT11.1* is positive indicated that the sensitive parent, BRRI dhan28 is responsible for this QTL. The second largest effect was observed in qPT2.1 with a LOD of 7.01 followed by *qPT3.1* with a LOD value of 6.11. Both the QTLs can explain about 12% phenotypic variation having negative additive effect that indicated the tolerant parent (Hasawi) was responsible for the QTLs.

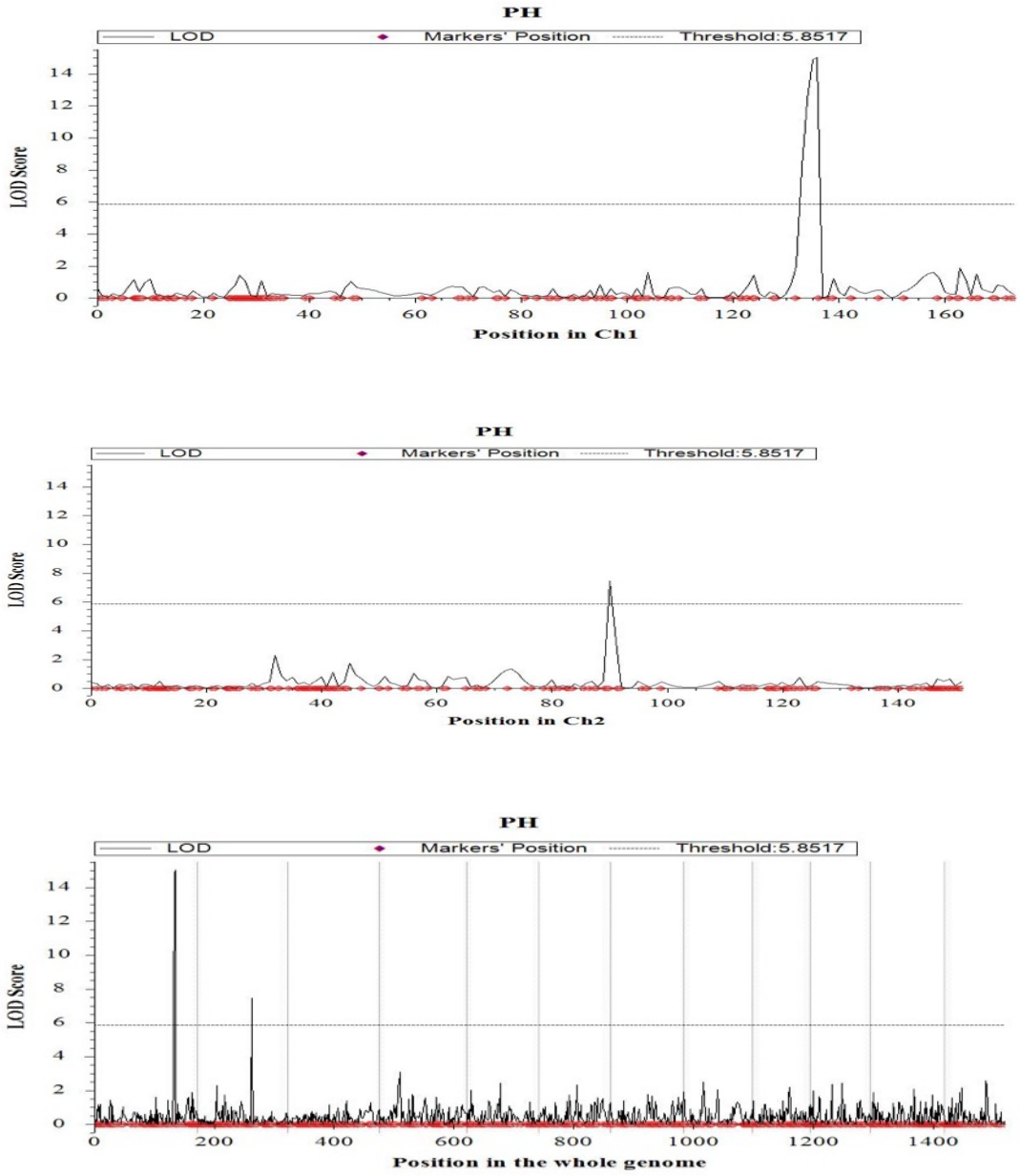


Figure 4.34. Chromosome locations of QTLs for plant height under salinity stress for 190 BC_1F_2 population from Hasawi x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) chromosome 1, (b) chromosome 2 and (c) on all chromosomes. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

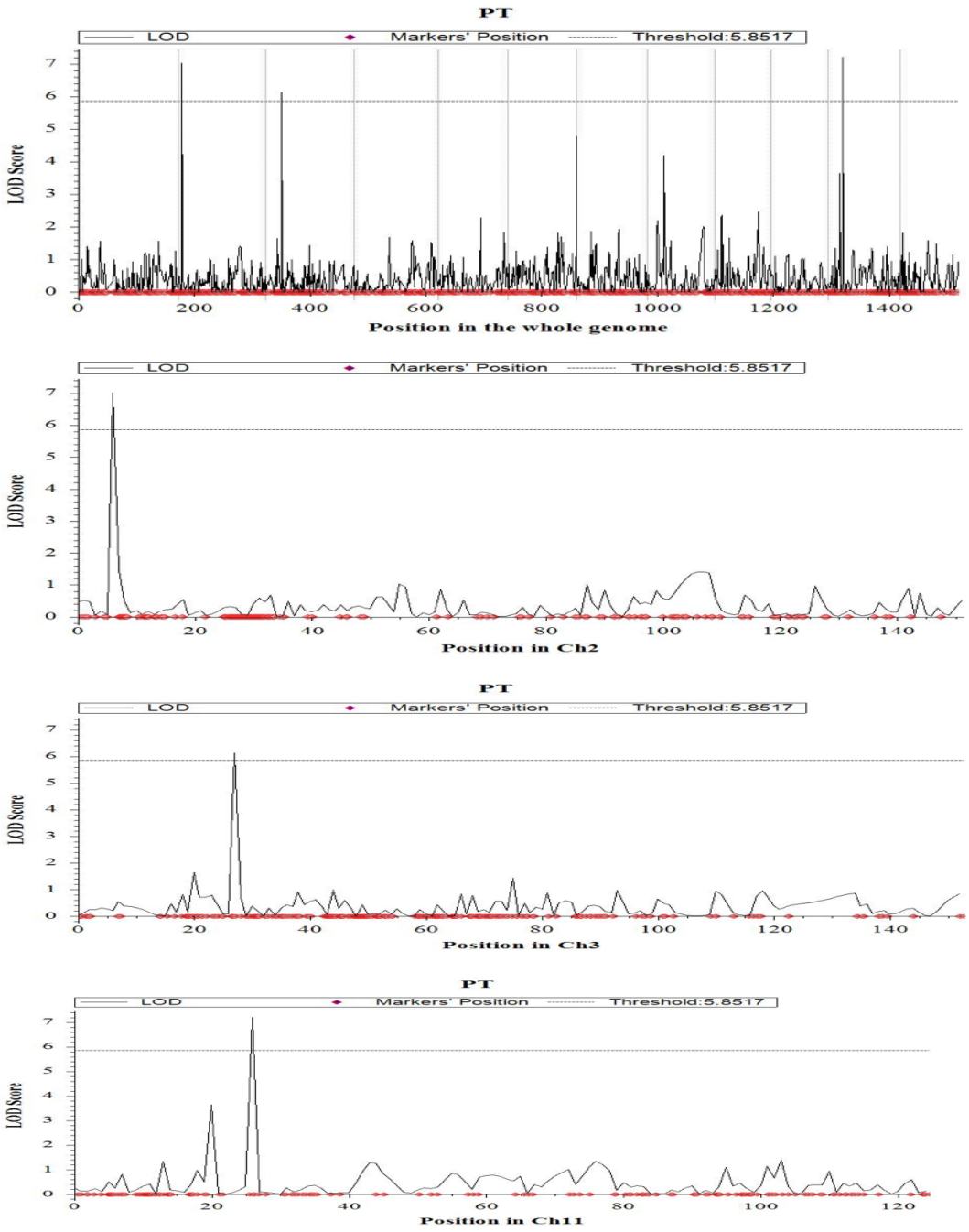


Figure 4.35. Chromosome locations of QTLs for productive tillers per plant under salinity stress for 190 BC_1F_2 population from Hasawi x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes, (b) chromosome 2, (c) chromosome 3 and (d) chromosome 11. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

4.4.4.4. Panicle Length

Seven QTLs were identified for panicle length on four chromosomes (chromosomes 2, 3, 6 and 12) (Table 4.3, Figs. 4.33 and 4.36). Among them, two QTLs (*qPL12.1* and *qPL6.1*) were significant ($p<0.05$) based on 1000 permutation. The largest effect of the significant QTLs was found in the short arm of chromosome 12 (*qPL12.1*) and long arm of chromosome 6 (*qPL6.1*) with LOD value of 8.64 and 6.36 and can explain 11.91% and 14.25% phenotypic variations, respectively. The QTL found in chromosome 12 showed the negative additive effect indicated that the tolerant parent, Hasawi is responsible for this QTL. In contrast, the QTL on chromosome 6 had positive additive effect indicating the sensitive parent, BRRI dhan28 contributed allele for this QTL. Among the QTLs which did not pass the 1000 permutation based significant test, three were identified on the long arm of chromosome 6 having the LOD value ranged from 3.09 to 4.01 and that explained 3.89% to 11% phenotypic variations. The rest two QTLs were identified on the long arm of chromosome 2 and short arm of chromosome 3 with a LOD value of 5.24 and 3.01 respectively.

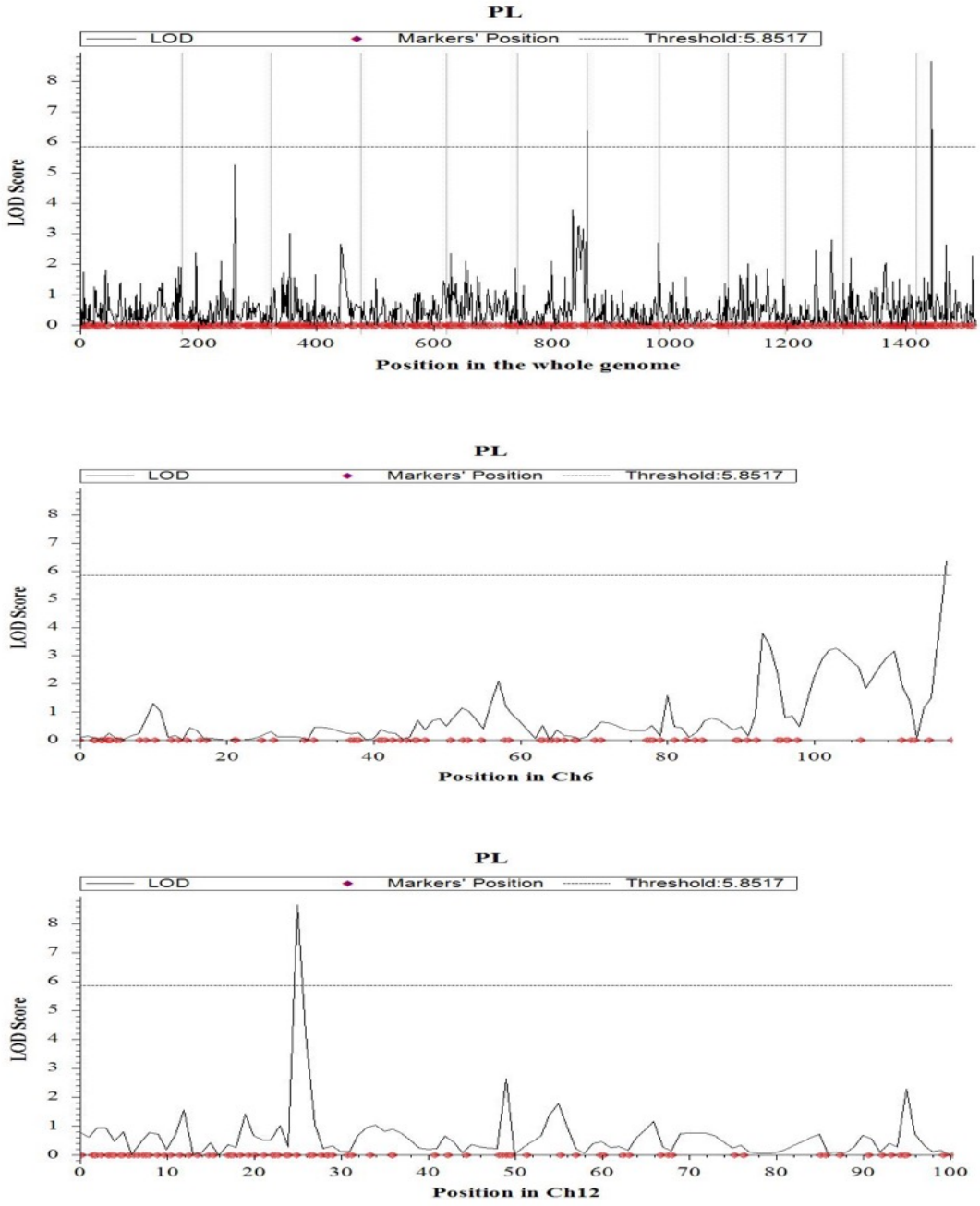


Figure 4.36. Chromosome locations of QTLs for panicle length under salinity stress for 190 BC_1F_2 population from Hasawi x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes, (b) chromosome 6 and (c) chromosome 12. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

4.4.4.5. Number of Filled Spikelets

Seven QTLs were identified for number of filled spikelets per plant on the chromosomes 3, 6, 7, 8, 11 and 12 (Table 4.3, Figs. 4.33 and 4.37). Among the QTLs, the largest effect was found in *qNFS3.1* with LOD value of 6.56 that can explain 17.94% phenotypic variation followed by qNFS7.1 with a LOD value of 3.48 that can explain 17.39% phenotypic variation. The QTLs were mapped on the short arm of chromosome 3 at 27.08 cM and chromosome 7 at 51.79 cM. In both cases, the additive effect was negative indicating the tolerant parent (Hasawi) is responsible for both the QTLs. The rest five QTLs were mapped in the long arm of chromosome 3, 6, 11 and 12 at 87.08 cM, 97.92 cM, 75.37 cM and 82.22 cM and one QTL on chromosome 8 was mapped at 4.22 cM in the short arm. Among all the QTLs, *qNFS8.1* and *qNFS6.1* showed positive additive effects indicating sensitive parent BRRI dhan28 contributed allele for both if this QTL.

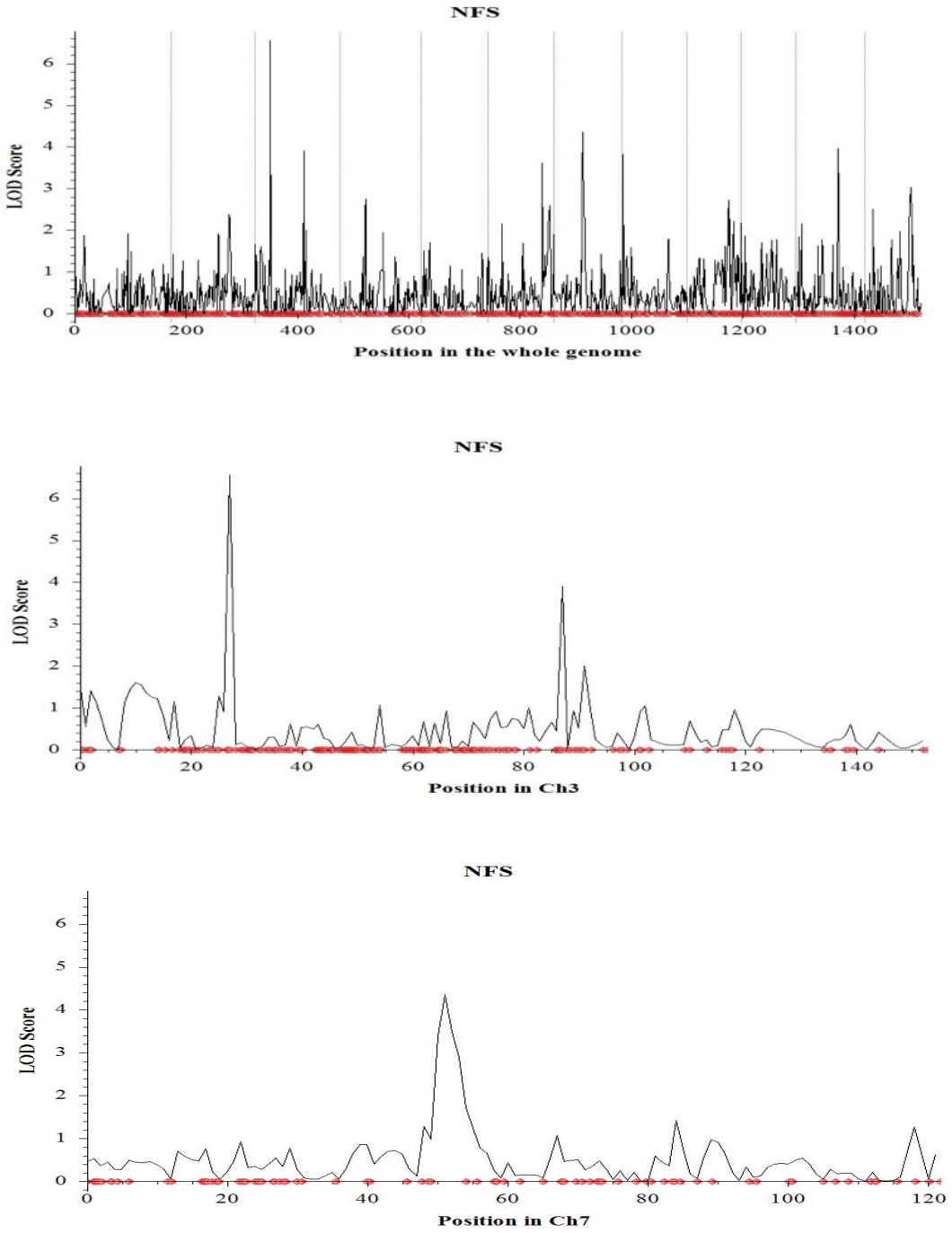


Figure 4.37. Chromosome locations of QTLs for number of filled spikelets per plant under salinity stress for 190 BC_1F_2 population from Hasawi x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes, (b) chromosome 7 and (c) chromosome 7. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

4.4.4.6. Number of Unfilled Spikelets

Only one QTL for number of unfilled spikelets per plant was identified on the short arm of chromosome 2 with a LOD value of 5.24 and can explain 11.42% phenotypic variation (Table 4.3, Figs. 4.33 and 4.38). This QTL exhibited negative additive effect indicated that the tolerant parent, Hasawi was responsible for the allele contribution.

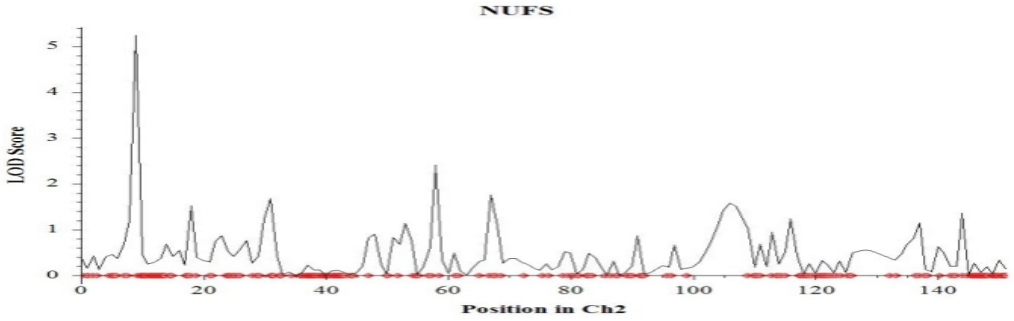


Figure 4.38. Chromosome locations of QTLs for number of unfilled spikelets per plant under salinity stress for 190 BC_1F_2 population from Hasawi x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

4.4.4.7. Percent Filled Spikelets

Three QTLs were identified for the percent filled spikelets on the chromosomes 4 and 6 with positive additive effects (Table 4.3, Figs. 4.33 and 4.39). Two QTLs (*qPFS4.1* and *qPFS6.1*) were significant ($p<0.05$) based on 1000 permutation and were mapped in the short arm of chromosome 4 at 9.36 cM and long arm of the chromosome 6 and at 111.92 cM with LOD value of 4.27 and 7.81 that explains about 15% phenotypic variations, respectively. The percent filled spikelets exhibited positive additive effect for both the QTLs. This implies that the sensitive parent, BRRI dhan28 is responsible for percent filled spikelets.

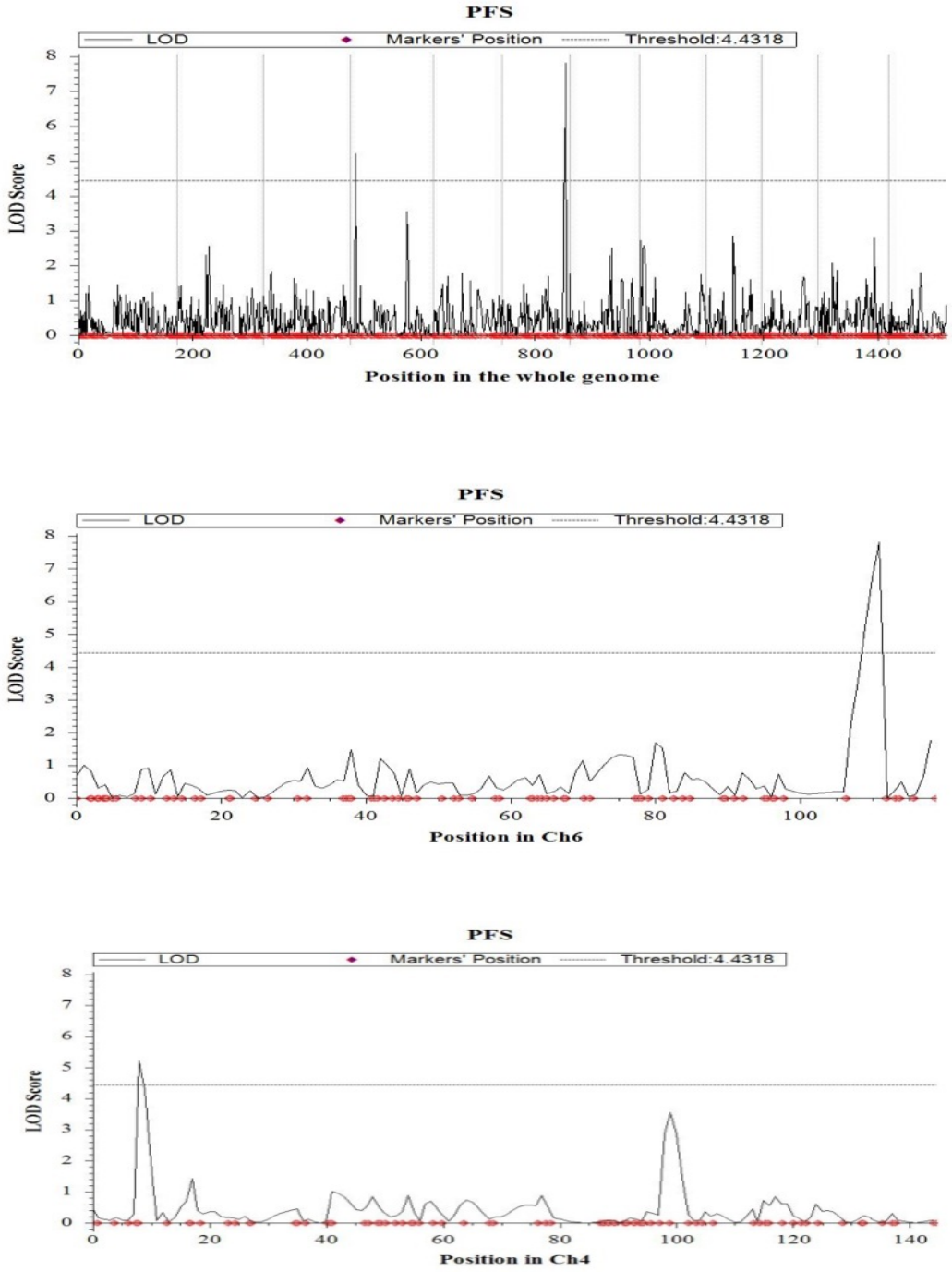


Figure 4.39. Chromosome locations of QTLs for percent filled spikelets under salinity stress for 190 BC_1F_2 population from Hasawi x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes, (b) chromosome 6 and (c) chromosome 4. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

4.4.4.8. Grain Yield

Eight QTLs were identified for the trait, grain yield per plant on the chromosomes 1, 3, 4, 6 and 9 (Table 4.3, Figs. 4.33 and 4.40) under salinity stress at the reproductive stage of rice. Among them, the largest effect was found in *qGY3.1* mapped on the short arm of chromosome 3 with LOD value of 7.30 that can explain 11.56% phenotypic variation followed by *qGY6.1* on chromosome 6 (mapped on short arm) with a LOD value of 7.06 that can explain 19.24% phenotypic variation and in *qGY1.1* on chromosome 1 (mapped on long arm) with LOD value of 6.18 which can explain 7.81% phenotypic variation. The additive effect was negative for two QTLs (*qGY3.1* and *qGY1.1*) indicating the tolerant parent (Hasawi) contributed alleles for both QTLs. But *qGY6.1* showed positive additive effects indicated the sensitive parent (BRRI dhan28) was responsible for contributing this QTL.

In addition, two more QTLs were found in the long and short arm chromosome 3 (*qGY3.2* and *qGY3.3*) at 87.08 cM and 2.08 cM; and *qGY6.2* were mapped in the long arm chromosome 6(Table 4.3). Rest two QTLs were identified on the long arm chromosomes 4 and 9 with LOD values of around 4.0 that explain 10.04% and 4.34% phenotypic variations, respectively.

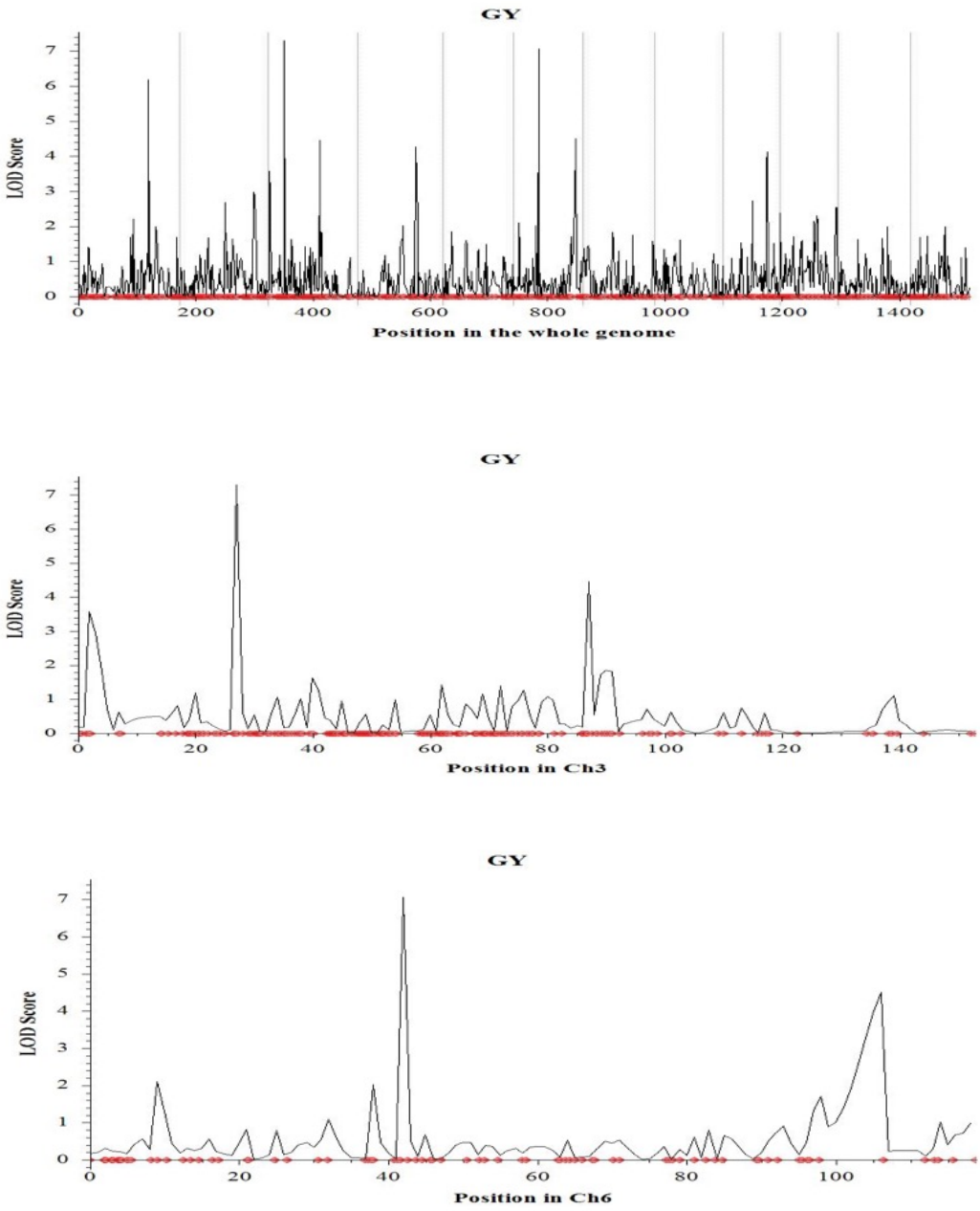


Figure 4.40. Chromosome locations of QTLs for grain yield per plant under salinity stress for 190 BC_1F_2 population from Hasawi x BRRI dhan28 based on significant threshold of LOD=3.0 using IciMapping (a) all chromosomes, (b) chromosome 3 and (c) chromosome 6. Horizontal line indicates the significant LOD threshold at 95% confidence levels based on 1000 permutations.

4.4.5. Comparison of New QTLs with Previous QTLs

In this study, three QTLs for plant height were identified on choromsome 1, 2 and 4. Of them two QTLs, *qPH1.1* and *qPH2.1* was mapped on the long arm of chromosome 1 and 2 at 139.67 cM and 91.23 cM, respectively. The tolerant parent, Hasawi contibuted alleles for both the QTLs. Mao et al., (2003) found an important dwarf QTL for plant height on chormosome 2 (qPH-2). The QTL idenfied in this study, *qPH2.1* lies within 100 kbp (4 cM) of the dwarf QTL identified by Mao et al., 2003.

Semi-dwarf 1 (*sd-1*) gene is the major gene of rice that revolutionized and significantly increased the yield of rice throughout the Asia during 1960s and onward. The phenotype of this gene is dwarfism which was the result from the deficiency of plant growth hormone, GA in the elongating stem . The location of this gene was on the long arm chomosome 1 and was mapped at 149.1 cM (Sasaki et al., 2002). In this study, *qPH1.1* was also idenfied on the long arm of chromosome 1 and was mapped at 138.67 cM, which is very near to the novel *sd-1* gene.

Haque et al., (2020); Mondal et al., (2019), Hossain et al., (2015) and Mohammadi et al., (2013) also observed similar. Haque et al. (2020) identified three QTLs for plant height on chormosome 1, 3 and 5. The QTL, *qPH.1@ 215* was mapped on the long arm of chromosome 1 at 215 cM and tolerant parent Horkuch contributed positive allele for this QTL. Although the identified QTL in this study was also on chromosome 1, it was located around 41 cM far from the QTL identified by Haque et al (2020). In addition, Mondal et al (2019) identified one QTL on chromosome 1 for plant height by using composite interval mapping (CIM) and tolerant parent BRRI dhan47 was responsible for this QTL. Hossain et

al., (2015) evaluated 218 F_2 individuals of Cheriviruppu (highly salt tolerant at both seedling and reproductive stage)/Pusa Basmati-1 (PB1) (sensitive at seedling and reproductive stage) at the reproductive stage using salinized water (EC 10 dS/m) and reported three significant QTLs on chromosomes 1, 4 and 7 for plant height. Cheriviruppu alleles contributed to the tallness of plants for all the QTLs. The long arm of chromosiome 1 contained a major QTL for plant height, *qPH-1.1s*, with 47.2% phenotypic variation. While, Mohammadi et al. (2013) obtained QTL for plant height on chromosome 1 (*qPH1.1s*), 2 (*qPH2.1s*), 3 (*qPH3.1s*) and 7 (*qPH7.1s*) using CIM with 232 F_2 population derived from the cross SADRI (tolerant at reproductive stage)/FL478 (sensitive at reproductive stage) using 6-8 dS/m salinity treatment. The loci had R^2 value ranging from 6.6 to 17.0 %. The QTL, *qPH1.1*s detected near the marker RM212 located on the long arm of chromosome 1 with the highest LOD value (9.4); the alleles from Sadri increased plant height.

Number of productive tillers per plant is also an important trait for determining the salinity tolerance in rice at reproductive stage. Six QTLs were identified in this study and among them the QTLs on chromosome 2 (*qPT2.1*), 3 (*qPT3.1*) and 11 (*qPT11.1*) crossed the threshold level ($p<0.05$) based on 1000 permutations. On chromosome 2, pollen killer gene; S29(t) was identified by Fengyi et al., (2006) from the BC_2F_2 mapping population derived from crosses between WAB450-6 and WAB56-104. The QTL, *qPT2.1* from the current study was located within 100 kbp region of this gene.

The most important findings from this study is that three QTLs, one each for productive tillers (*qPT3.1*), number of filled spikelets (*qNFS3.1*) and grain yield (*qGY3.1*) was mapped at the same position (6.73 Mb or 26.07 cM) on chromosome 3. Zhang et al.,

(2005) and Zhenbo et al., (1997) identified QTLs on chromosome 3 by within the 100 kbp region. Zhenbo et al., (1997)identified a QTL on grain yield (Gy3a) from double haploid population with a LOD value 3.11 that explained 12.1% phenotypic variations and mapped at around 5.5 Mb or 21.9 cM which is very near to the identified QTLs (*qPT3.1*, *qNFS3.1* and *qGY3.1*). But the yield QTL (Gy3a) was related to rationing ability of rice under non-stress conditions, not related to salinity stress. Zhang et al., (2005) identified a new sterile gene S34(t) in this region from BC_7F_1 mapping population of the crosses between IRGC103977 (*O. glaberrima*) and Dianjingyou 1 (*O. sativa*). As *O. glaberrima* has several sterile loci on chromosome 6, 11, 10 3 7 and 2. When single sterile gene or pyramiding of several sterile genes were transferred to Asian cultivar as a bridge to cross African species again, the F_1 was still high sterile. Basically, semi-sterility plants were used to make further backcross.

Oh et al., (2004) detected spikelet fertility QTL for cold tolerance, fer11 on chromosome 11 at 4.8 Mb positions; while *qPT11.1*of this study was identified for productive tiller on the same chromosome at 6.4 Mb position. Mondal et al., (2019) detected QTL on chromosome 11 for total tillers at 10.9 cM, which was far away from *qPT11.1* (located at 26.07 cM). None of the QTL for productive tillers was found on chromosome 2, 3 and 11 for salinity tolerance at respective location. So, these QTLs could be considered as novel QTLs.

For the panicle length, seven QTLs were identified, among them QTL peaks on chromosome 6 and 12 passed the significant threshold level ($p<0.05$) based on 1000 permutations (Zhuang et al., 2001) compared yield related traits between two mapping

population F_2 and RIL. The QTL for number of panicles for RIL was identified on chromosome 6 with LOD value of 2.02, located at around 27.3 Mb or 109.15 cM. Among the seven QTLs, qPT6.1 identified in this study was mapped at 118.92 cM, which is very near to the QTL identified by (Zhuang et al., 2001).

Number of filled spikelets per plant is one of the most important traits for grain yield, especially salinity tolerance particularly at the reproductive stage in rice. In this study, seven QTLs for this trait were detected on chromosome 3, 6, 7, 8, 11 and 12. Among them the largest effect of the QTL, *qNFS3.1* was found on chromosome 3 at 27.08 cM and tolerant parent, Hasawi contributed allele for this QTL. Haque et al., (2020) detected QTL for the number of filled grains on chromosome 10 at 58.48 cM, Mondal et al., (2019) identified single QTL on chromosome 2 and Mohammadi et al., (2013) detected four QTLs on chromosomes 2, 4, 6 and 10 for this trait using 232 F_2 populations of SADRI (tolerant)/FL478 (sensitive) at reproductive stage. So far, no QTL for this trait was found on chromosome 3. Therefore, *qNFS3.1* identified in this study could be considered as a novel QTL.

In this study, *qNFS6.1* explains 11.51% phenotypic variation having LOD value of 3.61 and was mapped at 91.91 cM on the long arm of chromosome 6; whereas the QTL *qFRSP6.1s* identified by Mohammadi et al., (2013) was mapped near the marker RM275 on the short arm of chromosome 6 which contributed 7.7% of the phenotypic variation. Although both the QTLs were found on the same chromosome; but the LOD values, phenotypic variations and positions of the QTLs identified were different. This may be due to the crossing of different parents with different salinity treatments. Percent filled spikelet

is also one of the most important yield components and is a good indicator for determining salinity tolerance in rice. Generally, due to salinity stress, percentage of filled spikelet will be reduced. But when the percent filled spikelets are moderate to high, the population is considered relatively tolerant. In this study, three QTLs were found on chromosome 4 and 6 among them, *qPFS4.1* and *qPFS6.1* crossed the threshold level ($p<0.05$) based on 1000 permutations. The QTL, *qPFS4.1* was identified on the short arm of chromosome 4 at 9.36 cM with LOD value of 4.27 and showed phenotypic variation of 14.29%. Similar findings were observed by Mei et al., (2006) from the reciprocal introgression line (IL). They also found QTL on chromosome 4 (*qSNP-4a*) at 2.71 cM with 16.07% phenotypic variation of 16.68 LOD value. Another study by Xiao et al., (1996) on RIL population identified QTL on chromosome 4 at 4.55 cM, which is relatively near to the identified QTL. Mondal et al., (2019) and Mohammadi et al. (2013) found QTL on chromosome 2 but at different positions. In addition, Jubay, (2012) identified three QTLs on chromosome 1, 4 and 12 for this trait. In this study, *qPFS6.1* identified on the long arm of chromosome 6 and was mapped at 111.92 cM; which was very close to the QTL for 1000 grain weight identified by Cho et al. (2008).

Number of unfilled spikelets tends to be increased due to salinity stress and is considered an important parameter to know the stress and magnitude of salinity on the performance of a crop. In this study, one QTL was identified on the short arm of chromosome 2 (*qNUFS2.1*) at 10.23 cM with the LOD value of 5.25 that explained 11.42% phenotypic variations. Ueda et al., (2013) reported similar results and they isolated pollen-detective mutant, Collapsed Abnormal Pollen 1 (CAP1) from insertional mutant lines of rice. This gene was located on the short arm chromosome 2 at 8.92 cM. Although the identified

QTLs were not similar to the current study, but Sabouri& Biabani, (2009) identified a good QTL *(qUFG-1b)* that showed the largest effect with 22.58% phenotypic variations.

Grain yield is the ultimate factor in determining salinity tolerance at reproductive stage of rice. The current study detected eight QTLs on chromosome 1, 3, 4, 6 and 9. Among them, QTL on chromosome 3 (*qGY3.1*) showed the largest effect with LOD value of 7.31, followed by QTL on chromosome 1 (*qGY1.1*) with LOD value of 6.18. The positions of the QTL were at 27.08 cM and 123.67 cM, respectively. Another QTL was also detected for grain yield on chromosome 1 with 8.41% phenotypic variation and 3.66 LOD value using 164 RIL population of Milyang 23/Gihobyeo by (Cho et al., 2007). Both QTLs were located on the long arm of chromosome 1. Jubay (2012) detected QTLs for grain yield on chromosomes 1, 3, 4 and 9 similar to the current study. But LOD value, position and phenotypic variations were different due to different salinity stress was imposed on different mapping population. Haque et al., (2020) detected QTL on chromosome 10 (qFGW.10@58 * Cyto) which was dissimilar with this study.

4.5. Summary and Conclusion

In this study, 588 BC_1F_2 population derived from the cross between a sensitive variety, BRRI dhan28, and a salt-tolerant variety, Hasawi, were evaluated under a salinity stress of EC 10 dS/m to identify quantitative trait loci (QTLs) for salinity tolerance at the reproductive stage of rice. Continuous 20 days salt stress was applied at the reproductive stage to 435 BC_1F_2 progenies and the rest 153 progenies were grown under non-stress (control) condition. Among 435 progenies, 28% were classified as tolerant, and the rest as sensitive based on their SES score and grain yield. About 45% each of the extremely tolerant

and highly sensitive progenies were used for QTL mapping. The findings and conclusions drawn from this study are summarized below:

The agronomic and yield related traits evaluated for salinity stress were plant height (PH), productive tillers (PT), panicle length (PL), number of filled spikelets (NFS), number of unfilled spikelets (NUFS), percent filled spikelets (PFS), percent unfilled spikelets (PUFS) and grain yield (GY). In addition, a physiological trait, sodium-potassium (Na^+/K^+) ratio was also evacuated. Almost all the traits differed significantly under salinity stress over those obtained under non-stress condition, except the percent filled grains. But all the traits of the tolerant and sensitive progenies under salt stress differed significantly.

Yield reduction between tolerant progenies of Hasawi x BRRI dhan28 grown under salinity stress and control condition was only 8.6%. The correlation analysis indicated positive and significant ($p<0.001$) correlation between grain yield and the number of filled spikelets, percent filled spikelets and productive tillers and significantly negative ($p<0.001$) correlation with SES score and percent unfilled spikelets of the progenies.

A total of 35 QTLs related to agronomic and physiological components under salinity stress were identified for all traits through inclusive composite interval mapping (ICIM) of Hasawi x BRRI dhan28 using IciMapping software 4.2. Total size of the genetic linkage map was 1556.57 cM, constructed using 6209 SNP markers. Most of the QTLs identified for the traits productive tillers, panicle length, no filled spikelets and grain yield and located on chromosome 3 and 6. But no QTLs were found on chromosome 5 and 10 for this cross.

In Hasawi x BRRI dhan28, eight QTLs for grain yield, seven QTLs each for panicle length and number of filled spikelets, six QTLs for productive tillers, three QTLs each for plant height and percent filled spikelets and a solo QTL for the number of unfilled spikelets were identified. Both tolerant (Hasawi) and sensitive (BRRI dhan28) parents contributed alleles to the QTLs for all traits except the plant height. All the data were statistically confirmed with permutation analysis of 1000 times at 5% level of significance. Out of 48 QTLs in Hasawi x BRRI dhan28; two each for plant height, panicle length and percent filled spikelets, and three QTLs for productive tillers were statistically confirmed. The QTLs identified in this study for reproductive-stage salt tolerance need to be fine mapped before they can be directly used to accelerate marker-assisted selection in future breeding programs to increase selection efficiency. The identification of the genes constituting these major QTLs would help to understand the molecular mechanisms.

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5. VALIDATION OF GRNA DESIGN FOR GENE EDITING OF HKT FAMILY OF TRANSPORTERS THROUGH CRISPR/Cas9 RIBONUCLEOPROTEINS

5.1. Introduction

Rice (*Oryza sativa* L.) is the main staple for more than three billion people of the world and it provides 20% dietary energy of the community depends on rice (Birla et al., 2017). However, various abiotic stresses, especially salinity greatly affects rice yield (Ahmadizadeh et al., 2016; Mondal et al., 2019). Rice plant is highly sensitive to the higher concentrations of sodium and its uptake causes Na^+ toxicity and osmotic stress (Horie et al., 2009). Therefore, development of salt tolerant rice varieties is the utmost need to increase rice production to feed the people living in the saline-prone environments of the world.

Several Na^+ transporters play an indispensable role in Na^+ tolerance in plants. Among them, HKT is a promising salt tolerant gene and it plays a fundamental role in both mono and dicotyledonous plants in the tolerance to salinity stress especially in rice (Almeida et al., 2013). The members of the HKT gene family in rice are expressed in xylem parenchyma cells and protect leaves from salt stress by removing sodium from the xylem sap (Platten et al., 2006). There are seven gene families reported for this HKT gene; indicates that the members of the HKT gene family play a central role in controlling Na^+ accumulation and also determine the mechanisms of salinity tolerance.

The recent advances in biotechnology and molecular breeding have brought tremendous changes in rice productivity across the globe with the development of improved varieties (Mishra et al., 2018). Genome editing with CRISPR/Cas 9 plays a vital role in improvement and understanding the gene functions associated with abiotic stresses in rice

and provides opportunity for plant improvement. Over the past 4 years the CRISPR/Cas 9 system has been widely adopted in rice, wheat, maize, soybean, potato etc. mainly for generating mutants (Liang et al., 2018). Further, CRISPR/Cas 9 system become more powerful with the introduction of multiplex genome editing which can target multiple genes with single gRNA or express multiple gRNA from single transcript. Among them, multiplex genome editing with the polycistronic tRNA-gRNA (PTG) approach presents a novel opportunity to create mutations in several members of the HKT gene family and to observe the changes in gene functions and trait phenotypes. This PTG approach is one of the simplest ways to assemble multiple gRNAs and later tRNA is excised by RnaseP and RNaseZ which is readily available in the cellular component (Xie et al., 2015). The CRISPR/Cas 9 components i.e. binary vector with desired PTG sequence, can be delivered into the plant cell via *Agrobacterium*-mediated delivery.

Delivering DNA in the form of a plasmid vector or delivering of mRNA itself can lead to expression of the Cas9 protein inside of a target cell and resulted in Cas9-mediated gene editing. Agrobacterium-mediated transformation has been extensively used for delivering genome-editing reagents. But due to enhancement of off-target cleavage and other foreign elements could be integrated into the mutants; this method limits the adoption in plant breeding.

To achieve CRISPR-mediated gene editing for HKT gene family, there must be a functional Cas9–gRNA ribonucleoprotein (RNP) complex present inside the nucleus and direct delivery of this RNP complex could be the most up-front option. For overcoming this problem of Agrobacterium mediated transformation, efficient DNA free genome editing method using CRISPR/Cas9 ribonucleoproteins (RNPs) has already been introduced as it

has less chance of having off-target mutations; possibly no off-target mutations detected in the mutant plants because of not using foreign DNA. This is also most difficult delivery format due to the large size and charge of the protein, but recently successful delivery of Cas9 protein has been demonstrated in vitro and in vivo. As a result, transgene free mutants could be obtained. This method may be widely applicable for producing genome edited crop plants and has a good prospect of being commercialized.

5.2. Objective

The general objective of this study is the designing an approach for multiplex CRISPR/Cas9 based gene editing targeting seven gene members of HKT family of sodium transporters. The specific activities were:

a. Sequence analysis of the HKT gene family in rice and designing appropriate gRNAs for the particular gene sequence of each member or conserved regions across multiple genes.

b. Validating the gRNA designs using *in vitro* ribonucleoprotein (RNP) assays by cleaving PCR amplicons containing the target site.

5.3. Materials and Methods

5.3.1. Plant Materials

In this study, rice (*Oryza sativa* L. ssp) cultivars, Presidio was used as a test variety and this was chosen because of its exceptional milling qualities, grain appearance and high yielding variety (USDA ARS, 2018). Rice plants were grown in a greenhouse of Texas A&M University with 12 h of light having the day and night temperature of 28 °C and 23 °C, respectively.

5.3.2. Selection of the Gene Family

High-Affinity K^+ transfer (HKT) gene family was selected for this study. Phylogenetic trees of HKT coding or amino-acid sequences show that this gene family classified into two major branches; named subfamily 1 and subfamily 2 (Platten et al., 2006). These two major subfamilies can be distinguished based on their gene organization i.e. introns are larger in the genes of subfamily 1 than subfamily 2 ($p = 0.0085$, Mann–Whitney test). Subfamily 1 contains four genes; OsHKT1;1 OsHKT1;3, OsHKT1,4 and OsHKT1;5 and subfamily 2 contains four genes; OsHKT2;1, OsHKT2;2 OsHKT2;3 and OsHKT2;4 (Platten et al., 2006). The reference sequence accession numbers were: OsHKT1;1, Os04g060750, OsHKT1;3, Os02g0175000, OsHKT1;4, Os04g0607600, OsHKT1;5, Os01g0307500, OsHKT2;1 Os06g0701700; OsHKT2;2 and OsHKT2;3, Os01g0532600.

5.3.3. Sequence Analysis of HKT Gene Family through Online Database

At first, the whole genome sequence of all seven genes was downloaded from the online database Rice Genome Annotation Project and identified the first exon (rice.plantbiology.msu.edu). Also, CDS sequence was downloaded from the same database. Then the CDS sequence of all seven genes were BLAST individually with nucleotide BLAST of NCBI. All of the genes had similar sequence with the Nipponbare rice genome sequence.

5.3.4. Primer Designing

A primer is a short nucleic acid sequence that provides a starting point for DNA synthesis. In living organisms, primers are short strands of RNA. A primer must be synthesized by an enzyme called RNA polymerase, before DNA replication can occur. For amplifying target gene of interest, primers are obvious to design for conducting PCR i.e. polymerase chain reaction and proper designing of primers ensure successful target DNA amplification (Cox, 2015). In the current study, most vital salt tolerant genes; HKT were used as a target gene. There are seven gene families in HKT gene and exon 1 region was selected as target region for all of them. Based on the target regions, seven pairs of primers were designed with the aid of software tool NCBI primer BLAST and also Primer3 (Xie et al., 2014).

At first, target sequences were downloaded from Gramene database and BLAST of exon 1 region was performed in NCBI for more accuracy of the sequence annotation. After that, target sequences were ready to use for primer designing (Xie et al., 2014). After pasting the target sequence and modifying the product size into 500 to 700 base pairs, possible

primers were achieved readily. The next step was to check the specificity of the primer pairs whether they are unique or not or they won't bind to other locations in the genome except the target (Xie et al., 2014). Aside from this; whether primer pairs bind to each other to form primer dimer, the possibility of the forming of secondary structure (like hairpin), melting temperature melting temperature (Tm), mismatch and tracking the annealing temperature (Ta) because if the temperature is too low, one or both primers will anneal to sequences other than the target region and lead to non-specific PCR amplification (https://www.idtdna.com/pages/tools/oligoanalyzer). Primer BLAST is one of the ways to check the primer specificity of the primer pairs by using Gramene database particularly to check comparatively less hits primers as those are considered good quality primers and IDT oligoanalyzer tools provides more information regarding Tm, Ta or formation of secondary structure

5.3.5. PCR Amplification of the Target Sequence

Rice genomic DNA of Presidio were extracted by CTAB method (Doyle, 1991) and target gene was amplified with specific primer pairs by using Phusion DNA Polymerase (Chester and Marshak, 1993). PCR reaction was performed to amplify the first exon of HKT gene family. It was carried out with 25 µl reaction mixture containing 2 µl of DNA having the concentration of 100 ng/µl, 4 µl of 5X Phusion high fidelity (HF) reaction buffer, 0.4 µl of 10mM dNTPs, 1 µl each forward and reverse primers of the HKT gene family, 0.2 µl of Phusion DNA polymerase and deionized water (Chester and Marshak, 1993, Xie et al., 2014). The standard reaction was initial denaturation at 94 C for 5 min followed by 32 cycles of denaturation at 94 C for 1 min, annealing at 61.2 for 1 min and extension at 72 C for 1 min, and final extension at 72 C for 7 min.

The PCR product was separated in 1% agarose gel and stained with cyber safe to identify the actual product size of the amplified PCR product. The stained gels were imaged using the Gel Doc XRS system (Bio-Rad). If the quality of the band was obscure and concentration of the sample is less (>20ng/ul); reamplification was done with the same primers. After image analysis, DNA concentration was measured with Nanodrop and gel extraction of the PCR product was done with Gel Extraction Kit (Xie et al., 2014). Lastly, the PCR products were sent to sequencing lab for getting the sequencing result which was based on dideoxy sequencing method DNA. After getting the sequencing result, Poly Peak Parsar online database/tool was used to analyze the sequencing result and based on the conserved region; highly specific gRNA was designed for each of the HKT gene family. CRISPR-direct; an online tool for gRNA design was used to design specific gRNA.

5.3.6. Assessment of the *in vitro* Cleavage Activity of CRISPR/Cas9 RNPs

Desired DNA fragments of OsHKT1;1, OsHKT1;3 OsHKT1;4 OsHKT1;5, OsHKT2;1 OsHKT2;3 and OsHKT2;4 were amplified through Polymerase Chain Reaction (PCR) by using target-specific primers designed through online database NCBI primer blast/Primer 3 and then run on an agarose gel to check the appropriate product size (Xie et al., 2014). After amplification, PCR product was purified with Gel Extraction kit and 30 µl of RNAse free water was added as final elution volume.

Reaction mixtures containing 3µl of 1µM Cas 9 protein, 3 µl of 300 mM gRNA (purchased from Synthego), 3 µl of Cas 9 NEB reaction buffer, and water was mixed thoroughly and kept it for 10 minutes at 25 ^{0}C (Larson et al., 2013, Mali et al., 2013,

Mehravar et al., 2019). After that Substrate DNA or PCR amplicons was added and incubated the whole reaction mixture at 37^{o} C for an hour.

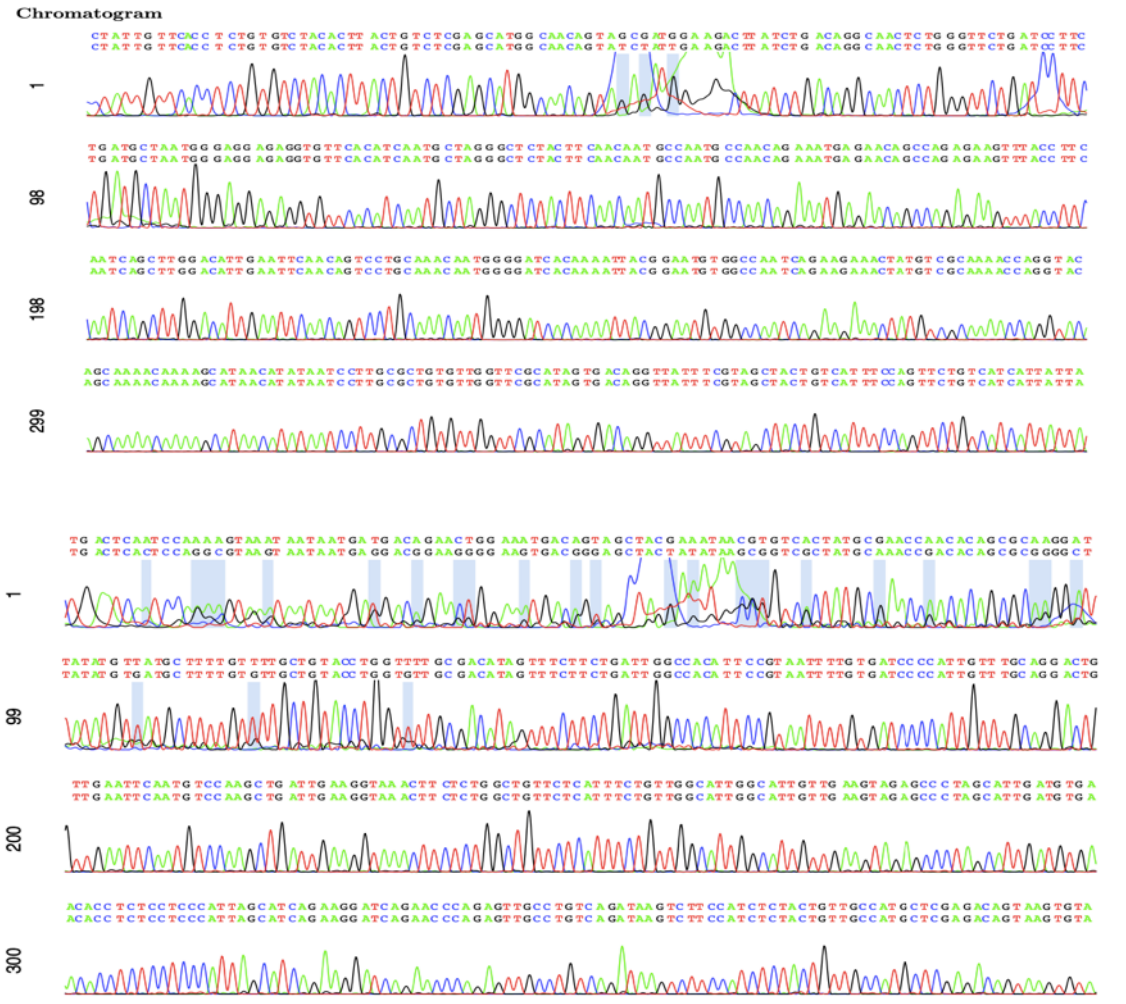
After an hour of incubation, 1 microliter of proteinase K was added into each of the 7 samples and mixed thoroughly with a pulse spin centrifuge (Larson et al., 2013, Mali et al., 2013, Mehravar et al., 2019). Then reaction mixtures were incubated for 10 to 20 minutes at 56 ^{0}C followed by running those samples in 2% agarose gel at 120 V for 20 minutes. Cleavage activity was analyzed by gel imager.

5.4. Results and Discussion

5.4.1. Analyzing of Sanger Sequencing Output

Sanger sequencing is the standard technology for sequencing as it provides a high degree of accuracy, long-read capabilities, and the flexibility to support a diverse range of applications in many research areas. It is highly recognized for DNA sequencing applications. PCR product of HKT gene family were sent out for sequencing to determine the correct gene sequences (bases) and their position in the genome. These sequences were compared with the reference sequence which was obtained from the Gramene database.

At very first chromatogram data was analyzed for every gene. In this study, online tool “Poly Peak Parser” was used to get the chromatogram data and it trims 30 base pairs from both 5’ and 3’ end to remove the low-quality bases. Best chromatogram data refers to have an evenly spaced nucleotide peaks and lack of baseline noise (Figs.5.1-5.7). Sometimes the computer software program mis-called a nucleotide, but it could be solved manually by scanning the sequences. Also, if there were any larger gap between the two peaks or two nucleotides, manually those sequences were scanned by looking over the sequences. In the current study, the chromatogram sequences of HKT gene families were reliable, because of having broader picks, basecall letters at the top was regular i.e. evenly spaced and lower noise.



Alignment

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                    ||||||||||||||||||||||||||||||||||||||||||||||||||| | || ||||||||||||||||||||||||
Reference        31 CTATTGTTCACCTCTGTGTCTACACTTACTGTCTCGAGCATGGCAACAGTAGCGATGGAAGACTTATCTGACAGGCAACT   110

Alt Allele       81 CTGGGTTCTGATCCTTCTGATGCTAATGGGAGGAGAGGTGTTCACATCAATGCTAGGGCTCTACTTCAACAATGCCAATG   160
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       111 CTGGGTTCTGATCCTTCTGATGCTAATGGGAGGAGAGGTGTTCACATCAATGCTAGGGCTCTACTTCAACAATGCCAATG   190

Alt Allele      161 CCAACAGAAATGAGAACAGCCAGAGAAGTTTACCTTCAATCAGCTTGGACATTGAATTCAACAGTCCTGCAAACAATGGG   240
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       191 CCAACAGAAATGAGAACAGCCAGAGAAGTTTACCTTCAATCAGCTTGGACATTGAATTCAACAGTCCTGCAAACAATGGG   270

Alt Allele      241 GATCACAAAATTACGGAATGTGGCCAATCAGAAGAAACTATGTCGCAAAACCAGGTACAGCAAAACAAAAGCATAACATA   320
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       271 GATCACAAAATTACGGAATGTGGCCAATCAGAAGAAACTATGTCGCAAAACCAGGTACAGCAAAACAAAAGCATAACATA   350

Alt Allele      321 TAATCCTTGCGCTGTGTTGGTTCGCATAGTGACAGGTTATTTCGTAGCTACTGTCATTTCCAGTTCTGTCATCATTATTA   400
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Alt Allele      401 TTTACTTTTGGATTGATTCAGATGCAAGAAATGTACTGAAAAGTAAGGAGATCAATA    457
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       431 TTTACTTTTGGATTGATTCAGATGCAAGAAATGTACTGAAAAGTAAGGAGATCAATA    487
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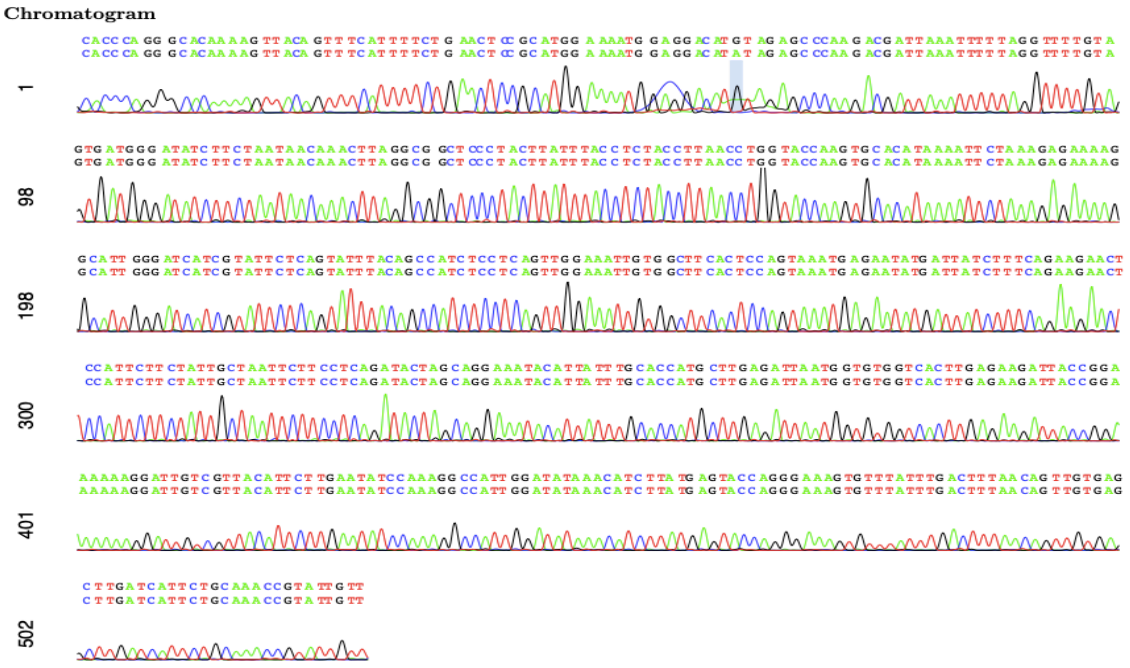
Figure 5.1. Chromatogram and alignment of OsHKT1;1



Alignment

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Alt Allele        1 CACCCAGGGCACAAAAGTTACAGTTTCATTTTCTGAACTCCGCATGGAAAATGGAGGACATATAGAGCCCAAGACGATTA    80
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Reference        31 CACCCAGGGCACAAAAGTTACAGTTTCATTTTCTGAACTCCGCATGGAAAATGGAGGACATGTAGAGCCCAAGACGATTA    110

Alt Allele       81 AATTTTTAGGTTTTGTAGTGATGGGATATCTTCTAATAACAAACTTAGGCGGCTCCCTACTTATTTACCTCTACCTTAAC    160
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Reference       111 AATTTTTAGGTTTTGTAGTGATGGGATATCTTCTAATAACAAACTTAGGCGGCTCCCTACTTATTTACCTCTACCTTAAC    190

Alt Allele      161 CTGGTACCAAGTGCACATAAAATTCTAAAGAGAAAAGGCATTGGGATCATCGTATTCTCAGTATTTACAGCCATCTCCTC    240
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       191 CTGGTACCAAGTGCACATAAAATTCTAAAGAGAAAAGGCATTGGGATCATCGTATTCTCAGTATTTACAGCCATCTCCTC    270

Alt Allele      241 AGTTGGAAATTGTGGCTTCACTCCAGTAAATGAGAATATGATTATCTTTCAGAAGAACTCCATTCTTCTATTGCTAATTC    320
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       271 AGTTGGAAATTGTGGCTTCACTCCAGTAAATGAGAATATGATTATCTTTCAGAAGAACTCCATTCTTCTATTGCTAATTC    350

Alt Allele      321 TTCCTCAGATACTAGCAGGAAATACATTATTTGCACCATGCTTGAGATTAATGGTGTGGTCACTTGAGAAGATTACCGGA    400
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Reference       351 TTCCTCAGATACTAGCAGGAAATACATTATTTGCACCATGCTTGAGATTAATGGTGTGGTCACTTGAGAAGATTACCGGA    430

Alt Allele      401 AAAAAGGATTGTCGTTACATTCTTGAATATCCAAAGGCCATTGGATATAAACATCTTATGAGTACCAGGGAAAGTGTTTA    480
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Reference       431 AAAAAGGATTGTCGTTACATTCTTGAATATCCAAAGGCCATTGGATATAAACATCTTATGAGTACCAGGGAAAGTGTTTA    510

Alt Allele      481 TTTGACTTTAACAGTTGTGAGCTTGATCATTCTGCAAACCGTATTGTT    528
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
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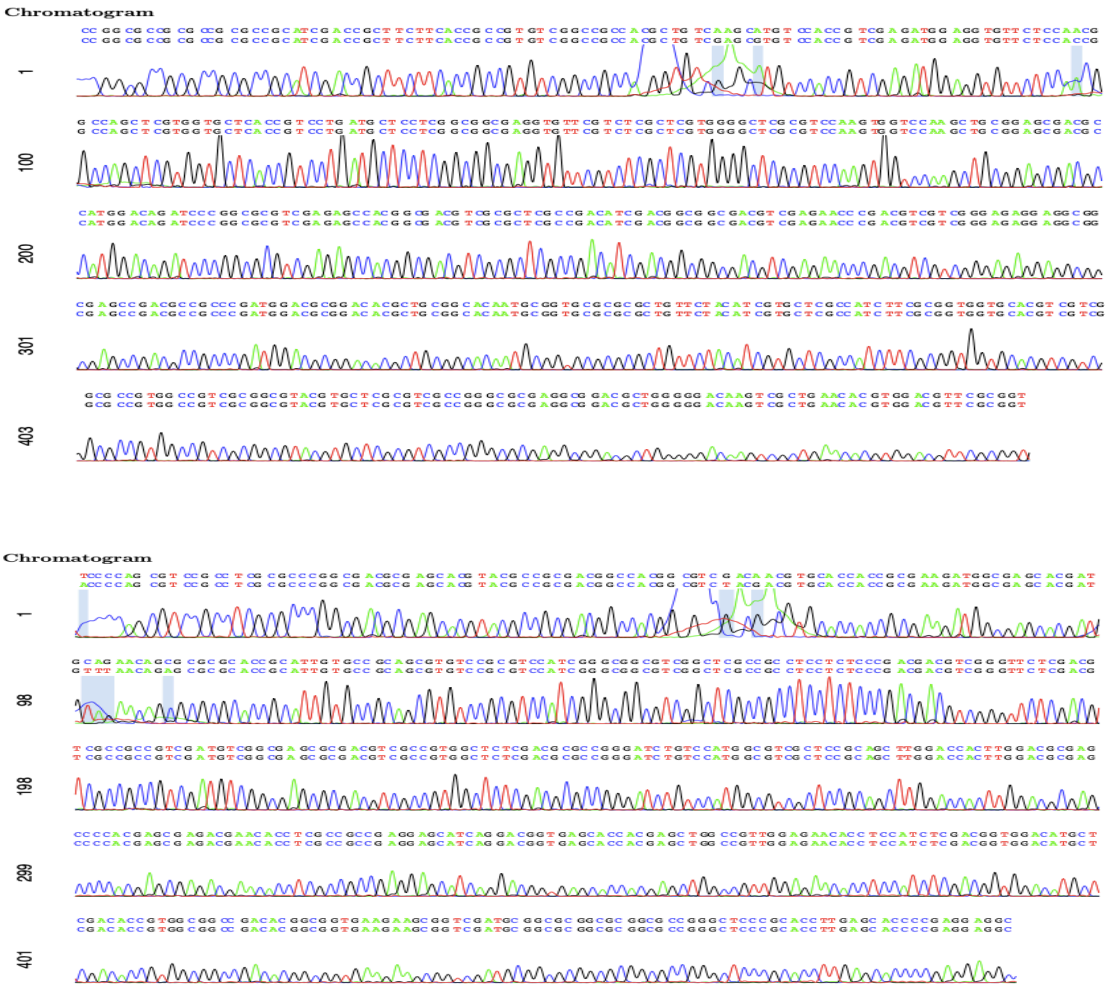
Figure 5.2. Chromatogram and alignment OsHKT1;3



Alignment

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Alt Allele        1 CCGGCGCCGCGCCGCGCCGCATCGACCGCTTCTTCACCGCCGTGTCGGCCGCCACGCTGTCAAGCGTGTCCACCGTCGAG    80
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Reference        31 CCGGCGCCGCGCCGCGCCGCATCGACCGCTTCTTCACCGCCGTGTCGGCCGCCACGCTGTCGAGCATGTCCACCGTCGAG   110

Alt Allele       81 ATGGAGGTGTTCTCCACCGGCCAGCTCGTGGTGCTCACCGTCCTGATGCTCCTCGGCGGCGAGGTGTTCGTCTCGCTCGT   160
                    ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       111 ATGGAGGTGTTCTCCAACGGCCAGCTCGTGGTGCTCACCGTCCTGATGCTCCTCGGCGGCGAGGTGTTCGTCTCGCTCGT   190

Alt Allele      161 GGGGCTCGCGTCCAAGTGGTCCAAGCTGCGGAGCGACGCCATGGACAGATCCCGGCGCGTCGAGAGCCACGGCGACGTCG   240
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       191 GGGGCTCGCGTCCAAGTGGTCCAAGCTGCGGAGCGACGCCATGGACAGATCCCGGCGCGTCGAGAGCCACGGCGACGTCG   270

Alt Allele      241 CGCTCGCCGACATCGACGGCGGCGACGTCGAGAACCCGACGTCGTCGGGGAGAGGAGGCGGCGAGCCGACGCCGCCCGATG   320
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       271 CGCTCGCCGACATCGACGGCGGCGACGTCGAGAACCCGACGTCGTCGGGGAGAGGAGGCGGCGAGCCGACGCCGCCCGATG   350

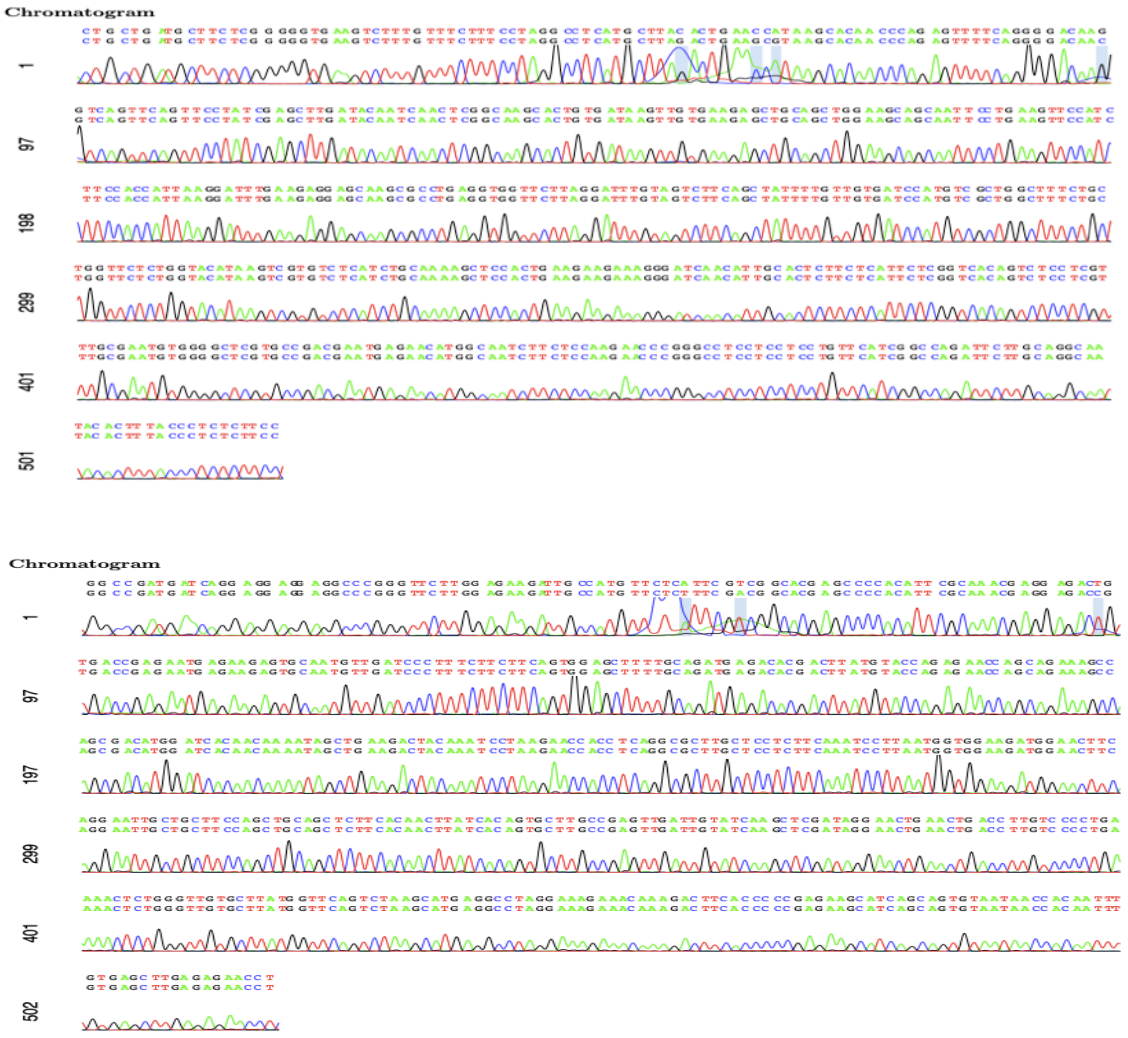
Alt Allele      321 GACGCGGACACGCTGCGGCACAATGCGGTGCGCGCGCTGTTCTACATCGTGCTCGCCATCTTCGCGGTGGTGCACGTCGT   400
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       351 GACGCGGACACGCTGCGGCACAATGCGGTGCGCGCGCTGTTCTACATCGTGCTCGCCATCTTCGCGGTGGTGCACGTCGT   430

Alt Allele      401 CGGCGCCGTGGCCGTCGCGGCGTACGTGCTCGCGTCGCCGGGCGCGAGGCGGACGCTGGGGGACAAGTCGCTGAACACGT   480
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference       431 CGGCGCCGTGGCCGTCGCGGCGTACGTGCTCGCGTCGCCGGGCGCGAGGCGGACGCTGGGGGACAAGTCGCTGAACACGT   510

Alt Allele      481 GGACGTTCGCGGT    493
                    |||||||||||||
Reference       511 GGACGTTCGCGGT    523
```

Figure 5.3. Chromatogram and alignment OsHKT1;4

Figure 5.4. Chromatogram and alignment OsHKT1;5



Alignment

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                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||| ||||||||||||||||||
Reference (revcomp)   31 GGCCGATGATCAGGAGGAGGAGGCCCGGGTTCTTGGAGAAGATTGCCATGTTCTCATTCGTCGGCACGAGCCCCACATTC    110

Alt Allele            81 GCAAACGAGGAGACCGTGACCGAGAATGAGAAGAGTGCAATGTTGATCCCTTTCTTCTTCAGTGGAGCTTTTGCAGATGA    160
                         ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference (revcomp) 111 GCAAACGAGGAGACTGTGACCGAGAATGAGAAGAGTGCAATGTTGATCCCTTTCTTCTTCAGTGGAGCTTTTGCAGATGA    190

Alt Allele            161 GACACGACTTATGTACCAGAGAACCAGCAGAAAGCCAGCGACATGGATCACAACAAAATAGCTGAAGACTACAAATCCTA    240
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference (revcomp) 191 GACACGACTTATGTACCAGAGAACCAGCAGAAAGCCAGCGACATGGATCACAACAAAATAGCTGAAGACTACAAATCCTA    270

Alt Allele            241 AGAACCACCTCAGGCGCTTGCTCCTCTTCAAATCCTTAATGGTGGAAGATGGAACTTCAGGAATTGCTGCTTCCAGCTGC    320
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Reference (revcomp) 271 AGAACCACCTCAGGCGCTTGCTCCTCTTCAAATCCTTAATGGTGGAAGATGGAACTTCAGGAATTGCTGCTTCCAGCTGC    350

Alt Allele            321 AGCTCTTCACAACTTATCACAGTGCTTGCCGAGTTGATTGTATCAAGCTCGATAGGAACTGAACTGACCTTGTCCCCTGA    400
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Alt Allele            481 GCAGTGTAATAACCACAATTTGTGAGCTTGAGAGAACCT    519
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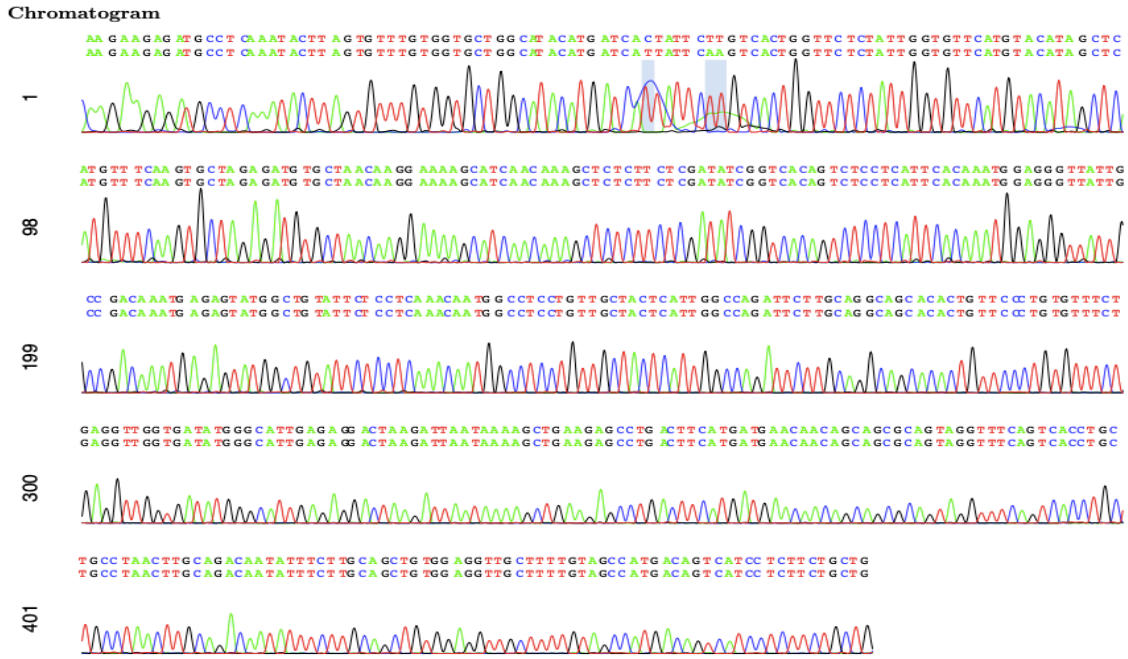
Figure 5.5. Chromatogram and alignment OsHKT2;1



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Reference        31 AAGAAGAGATGCCTCAAATACTTAGTGTTTGTGGTGCTGGCATACATGATCATTATTCTTGTCACTGGTTCTCTATTGGT    110

Alt Allele       81 GTTCATGTACATAGCTCATGTTTCAAGTGCTAGAGATGTGCTAACAAGGAAAAGCATCAACAAAGCTCTCTTCTCGATAT    160
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Reference       111 GTTCATGTACATAGCTCATGTTTCAAGTGCTAGAGATGTGCTAACAAGGAAAAGCATCAACAAAGCTCTCTTCTCGATAT    190

Alt Allele      161 CGGTCACAGTCTCCTCATTCACAAATGGAGGGTTATTGCCGACAAATGAGAGTATGGCTGTATTCTCCTCAAACAATGGC    240
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Reference       191 CGGTCACAGTCTCCTCATTCACAAATGGAGGGTTATTGCCGACAAATGAGAGTATGGCTGTATTCTCCTCAAACAATGGC    270

Alt Allele      241 CTCCTGTTGCTACTCATTGGCCAGATTCTTGCAGGCAGCACACTGTTCCCTGTGTTTCTGAGGTTGGTGATATGGGCATT    320
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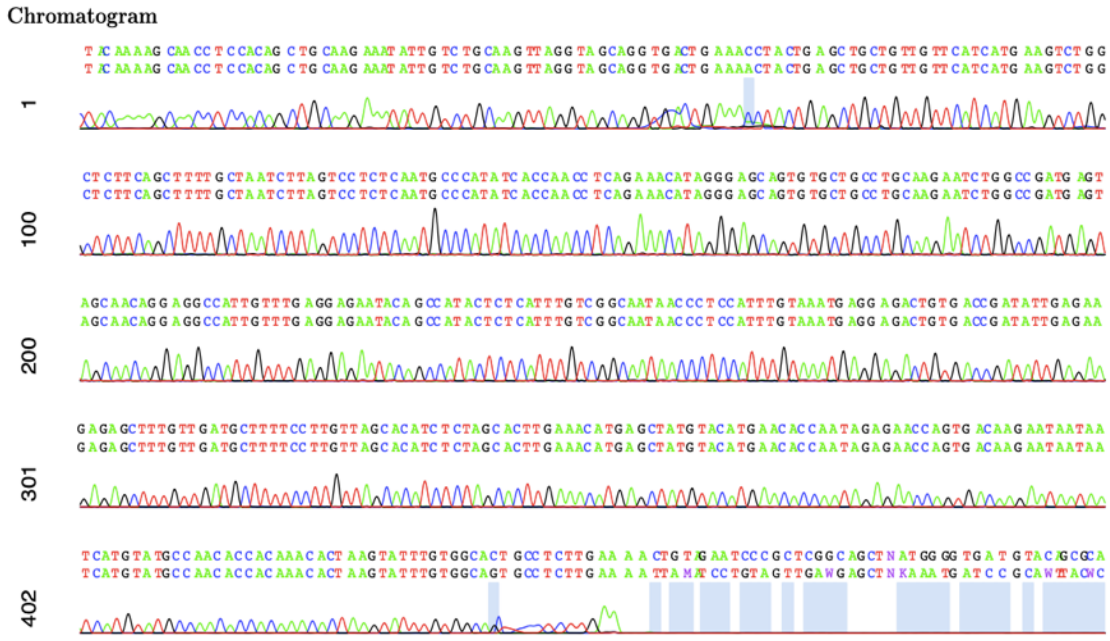
Figure 5.6. Chromatogram and alignment OsHKT2;3



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Reference (revcomp)  31 TACAAAAGCAACCTCCACAGCTGCAAGAAATATTGTCTGCAAGTTAGGTAGCAGGTGACTGAAACCTACTGAGCTGCTGT    110

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Reference (revcomp) 111 TGTTCATCATGAAGTCTGGCTCTTCAGCTTTTGCTAATCTTAGTCCTCTCAATGCCCATATCACCAACCTCAGAAACATA    190

Alt Allele          161 GGGAGCAGTGTGCTGCCTGCAAGAATCTGGCCGATGAGTAGCAACAGGAGGCCATTGTTTGAGGAGAATACAGCCATACT    240
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Reference (revcomp) 191 GGGAGCAGTGTGCTGCCTGCAAGAATCTGGCCGATGAGTAGCAACAGGAGGCCATTGTTTGAGGAGAATACAGCCATACT    270

Alt Allele          241 CTCATTTGTCGGCAATAACCCTCCATTTGTAAATGAGGAGACTGTGACCGATATTGAGAAGAGAGCTTTGTTGATGCTTT    320
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Reference (revcomp) 271 CTCATTTGTCGGCAATAACCCTCCATTTGTAAATGAGGAGACTGTGACCGATATTGAGAAGAGAGCTTTGTTGATGCTTT    350

Alt Allele          321 TCCTTGTTAGCACATCTCTAGCACTTGAAACATGAGCTATGTACATGAACACCAATAGAGAACCAGTGACAAGAATAATA    400
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Reference (revcomp) 351 TCCTTGTTAGCACATCTCTAGCACTTGAAACATGAGCTATGTACATGAACACCAATAGAGAACCAGTGACAAGAATAATA    430

Alt Allele          401 ATCATGTATGCCAACACCACAAACACTAAGTATTTGTGGCACTGCCTCTTGAAAA    455
                        ||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Reference (revcomp) 431 ATCATGTATGCCAACACCACAAACACTAAGTATTTGTGGCAGTGCCTCTTGAAAA    485
```

Figure 5.7. Chromatogram and alignment OsHKT2;4

5.4.2. Guide RNA (gRNA) Design of HKT Gene Family

The gRNA is a short synthetic RNA composed of a scaffold sequence necessary for Cas-binding and a user-defined 20 nucleotide spacer sequence that determines the genomic target to be modified. Designing gRNA was the primary work for CRISPR/Cas 9 Genome editing. Some important criteria were maintained to design gRNA. They were; GC content should be 40% to 60%, guide sequence should be 20 base pair in length, position and number of mismatches should not be near the seed region. There are a couple of online tools available for designing gRNA. For this study, 'CRISPR-direct' (Naito et al., 2015) and 'CRISPR-P' (Lei et al., 2014) was used to design gRNA. Exon 1 sequence of HKT genes families were used as target for designing gRNA and highly specific sequences were selected as target (Fig. 5.8). These gRNAs were ordered from "Synthego Corporation" as it offers high quality synthetic gRNA.

5.4.3. Targeted Cleavage of HKT Genes *in vitro*

As the first step, CRISPR/Cas9 RNPs were tested to check if the gRNAs properly cleave the targeted genomic sites or not. In the current study, all of the seven gRNAs cleaved the target regions of the HKT gene families. The total size of the PCR product for OsHKT1;1, OsHKT1;3, OsHKT1;4, OsHKT1;5, OsHKT2;1, OsHKT2;3 and OsHKT2;4 was 550 bp, 615 bp, 583 bp, 504 bp, 611 bp, 580 bp and 514 bp, respectively. But the cleavage site of the seven HKT genes was different from each other due to having different protospacer adjacent motif (PAM) site (Fig. 5.9-5.12). In this current study, only cleavage site was confirmed by the *in vitro* RNP test.

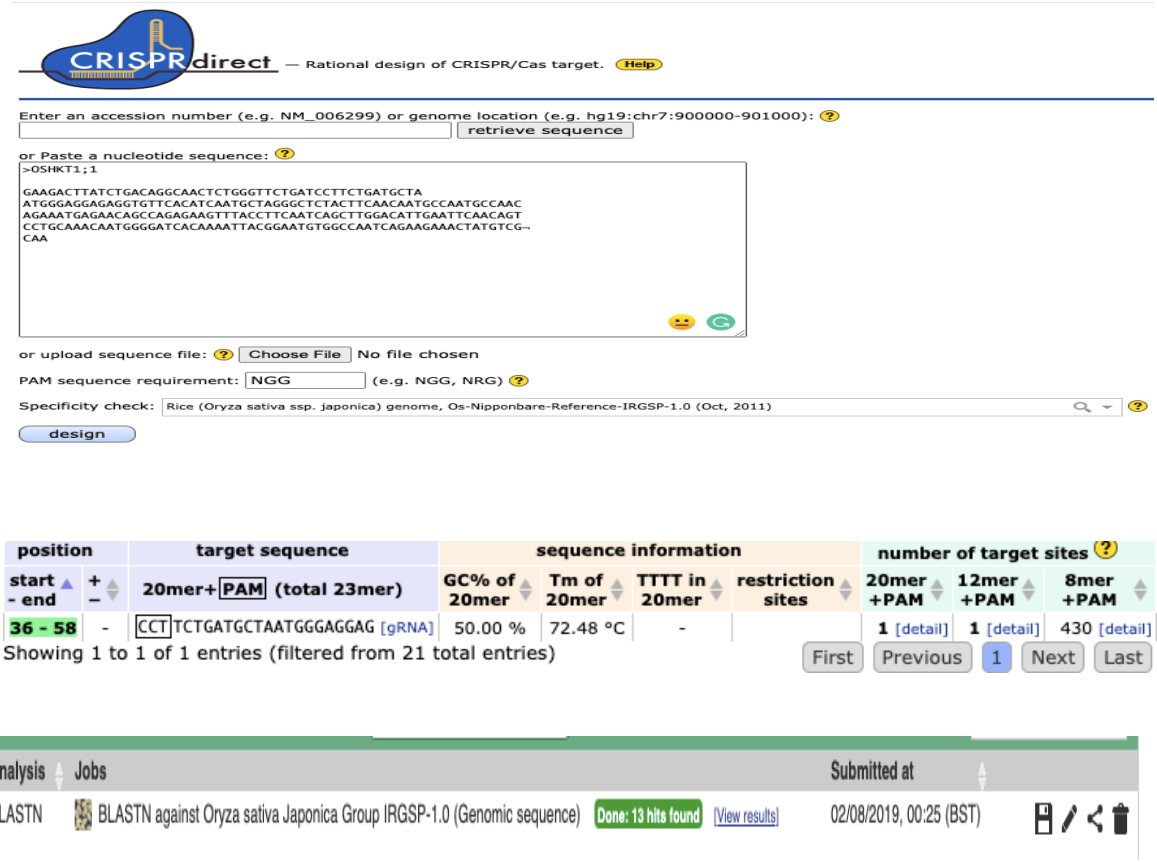

Figure 5.8. gRNA design by CRISPR-direct

The next step will be to go for protoplast assay or isolation to transform the RNP products into the isolated protoplast via PEG-mediated transformation. Then, the DNA will be isolated from the protoplasts by using an appropriate method possible CTAB method (Doyle, 1991) of DNA extraction and DNA concentration will be determined through a Nanodrop spectrophotometer. After DNA extraction, CRISPR/Cas9 RNP nuclease activity will be determined in the protoplast by the PCR/RE assay. Then the RNP will be delivered via gene gun or particle bombardment into the rice (Presidio) calli or shoot apical meristem (SAM).

Conventional breeding approaches (e.g. backcrossing) take a long time to develop a desired crop variety; therefore, working at the genomic level is a promising way to save time and resources to develop a new variety having desired characteristics. Multiplex genome editing could simultaneously target more than one gene and mutated plants can be produced by introducing CRISPR/Cas 9 plasmid into the plant cells. Moreover, a DNA-free genome editing system has been developed via Cas9-gRNA RNP complex to pre-validate the functionality and efficiency of CRISPR-Cas9 system for targeting specific genes like HKT (Liang et al., 2017, 2018; Mehravar et al., 2019). It is a very simple and rapid method of validation before delivering the genes into the particular organ like shoot apical meristem (SAM) or calli and no other CRISPR components need to deliver separately.

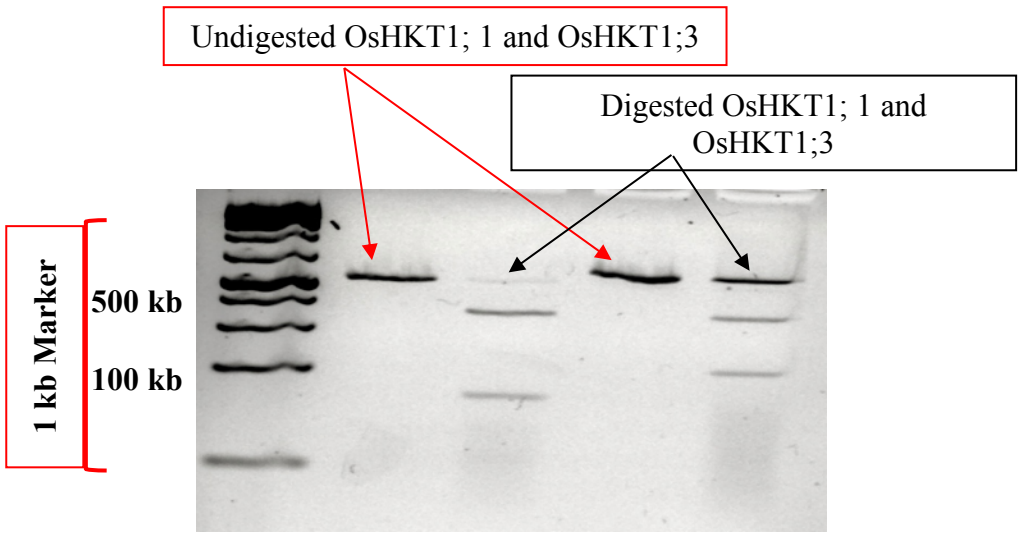

Figure 5.9. *in vitro* cleavage with Cas9 nuclease and gRNA; Lane 1: 1kb plus marker, Lane 2: uncut PCR product of OsHKT1;1, Lane 3: PCR product of OsHKT1;1+Cas9+gRNA, Lane 4: Uncut PCR product of OsHKT1;3 Lane 5: PCR product of OsHKT1;3+Cas9+gRNA.

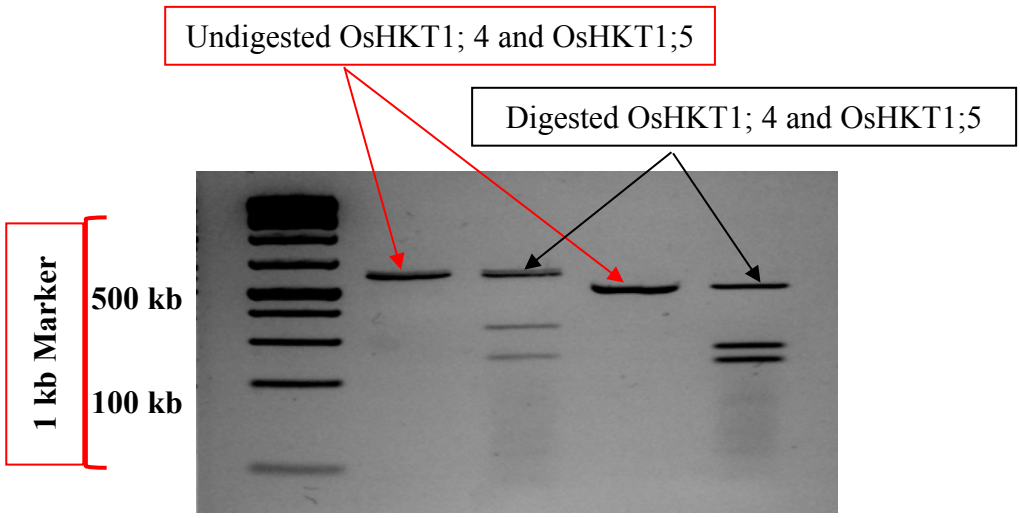

Figure 5.10. *in vitro* cleavage with Cas9 nuclease and gRNA; Lane 1: 1kb plus marker, Lane 2: uncut PCR product of OsHKT1;4, Lane 3: PCR product of OsHKT1;4+Cas9+gRNA, Lane 4. Uncut PCR product of OsHKT1;5 and Lane 5: PCR product of OsHKT1;5+Cas9+gRNA.

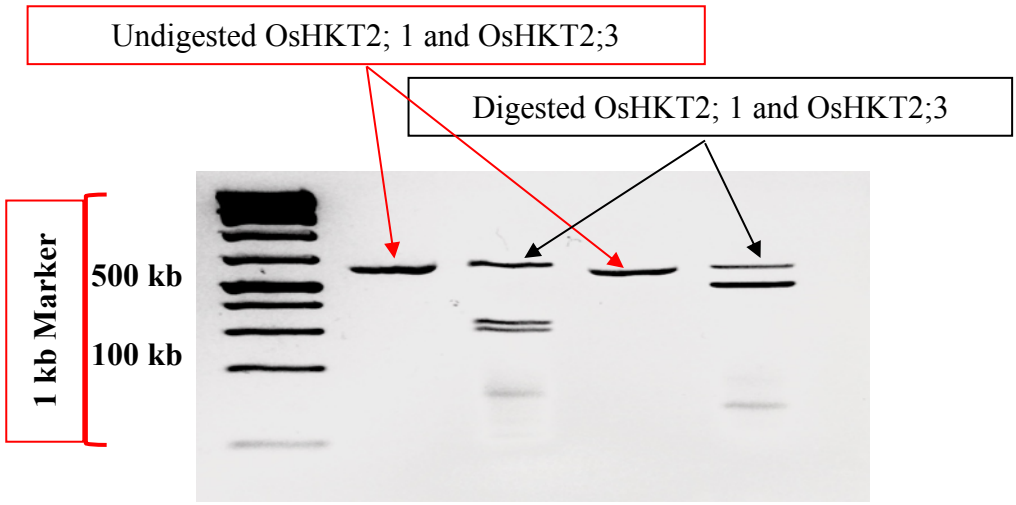

Figure 5.11. *in vitro* cleavage with Cas9 nuclease and gRNA; Lane 1: 1kb plus marker, Lane 2: uncut PCR product of OsHKT2;1, Lane 3: PCR product of OsHKT2;1+Cas9+gRNA, Lane 4. Uncut PCR product of OsHKT2;3 and Lane 5: PCR product of OsHKT2;3+Cas9+gRNA.

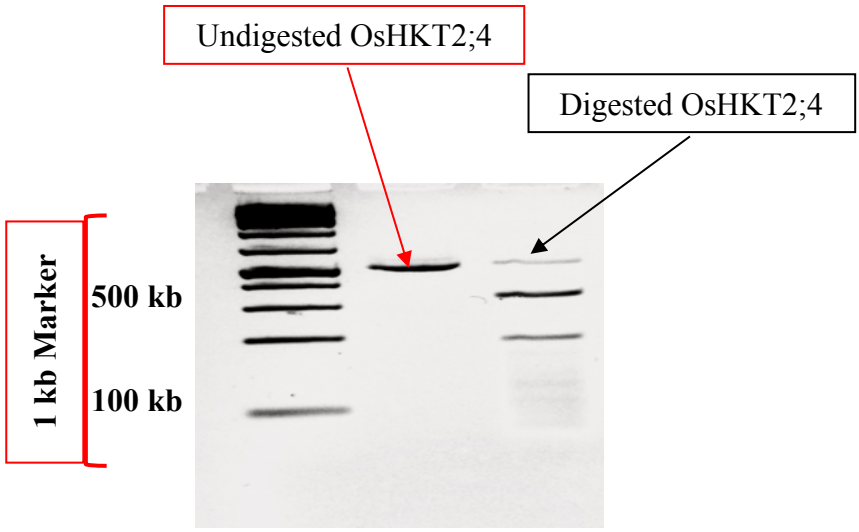

Figure 5.12. *in vitro* cleavage with Cas9 nuclease and gRNA; Lane 1: 1kb plus marker, Lane 2: uncut PCR product of OsHKT2;4, Lane 3: PCR product of OsHKT2;4+Cas9+gRNA.

5.5. Summary and Conclusion

Enhanced agricultural production through innovative breeding technology is urgently needed to feed the ever-growing population and improve their nutrition worldwide. The advancement in biotechnology and molecular breeding brought tremendous changes in agricultural productivity across the globe. Over the past few years, advances in CRISPR/Cas9 genome editing enabled efficient targeted modification in most agricultural crops and widely adopted in rice, wheat, maize, soybean, potato etc. mainly for generating mutants. Among the cereals, rice is grown and consumed widely. But due to climate change, abiotic stress like salinity posing threat to productivity. Since rice plant is highly sensitive to the higher concentrations of sodium and its uptake causes Na^+ toxicity and osmotic stress, it is imperative to understand the molecular mechanisms of salt tolerance genes particularly HKT genes to develop improved rice varieties for food security of the population living in the saline prone environment. Therefore, the present study was conducted to target seven gene members of HKT gene family of sodium transporters via multiplex CRISPR/Cas9 based DNA free genome editing that offers less off-target effects when using CRISPR/Cas9 RNPs because they are degraded rapidly after entering into the plant cells and the desired mutants can be readily obtained in the T_0 generation, which saves time and money.

In this study, appropriate gRNAs targeting seven gene members were designed and pre-validated by CRISPR-Cas9 ribonucleoprotein (RNP) complex. The results showed that, each of the genes was cleaved in an appropriate manner. The results suggest that such a delivery of CRISPR reagents into cells will ensure that no foreign DNA will integrate into the genome as the system relies on transient activity of the Cas9/gRNA complex. It is

indispensable to pre-validate the gRNA efficacy for in vivo applications such as delivery of CRISPR reagents through particle bombardment.

5.6. References

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6. CONCLUSIONS

Globally, salinity is the most prevalent and widespread problem in agricultural productivity. Rice is one of the most important cereal crops that feed about half of the world. The reproductive stage of the crop is sensitive to biotic and abiotic stress as it depresses grain yield much more than any other stages. In this study, two large number of BC_1F_2 population (588 and 624 lines) derived from the crosses between a sensitive variety, BRRI dhan28 and two salt tolerant varieties, CSR28 and Hasawi were evaluated under a salinity stress of EC 10 dS/m to identify quantitative trait loci (QTLs) for salinity tolerance at the reproductive stage of rice. Continuous 20 days salt stress was applied at the reproductive stage to 435 BC_1F_2 progenies each derived from the crosses, CSR28 x BRRI dhan28 and Hasawi x BRRI dhan28 and about 200 progenies were grown under non-stress (control) conditions. Among the salt-stressed progenies, 46% plants from CSR28 x BRRI dhan28 and 28% from Hasawi x BRRI dhan28 were classified as tolerant, and the rest as sensitive based on their SES score and grain yield. About 45% each of the extremely tolerant and highly sensitive progenies from both crosses were used for QTL mapping. The findings and conclusions drawn from this study are summarized below:

The agronomic and yield related traits evaluated for salinity stress were plant height (PH), productive tillers (PT), panicle length (PL), number of filled spikelets (NFS), number of unfilled spikelets (NUFS), percent filled spikelets (PFS), percent unfilled spikelets (PUFS) and grain yield (GY). In addition, sodium-potassium (Na^+/K^+) ratio was also evaluated. Almost all the traits differed significantly under salinity stress over those obtained under non-stress conditions, except the percent filled grains of Hasawi x BRRI dhan28, and

grain yield of CSR28 x BRRI dhan28. But all the traits of the tolerant and sensitive progenies for both the crosses under salt stress differed significantly.

Both the percent filled grains and its reduction due to salinity stress were higher among the progenies of Hasawi x BRRI dhan28 than CSR28 x BRRI dhan28. The grain yield of tolerant progenies from the cross, CSR28 x BRRI dhan28 grown under salinity stress differed significantly over the progenies grown under control condition, but the yield difference was not significant among the progenies of Hasawi x BRRI dhan28. Yield reduction between tolerant progenies of CSR28 x BRRI dhan28 and Hasawi x BRRI dhan28 grown under salinity stress and control condition was 15.8% and 8.6%, respectively indicated the superiority of the Hasawi x BRRI dhan28 progenies in developing salt tolerant varieties. The correlation analysis indicated positive and significant ($p<0.001$) correlation between grain yield and the number of filled spikelets, percent filled spikelets and productive tillers and significantly negative ($p<0.001$) correlation with the SES score and percent unfilled spikelets of the BC_1F_2 progenies derived from both the crosses.

A total of 15 and 48 QTLs related to agronomic and yield components under salinity stress were identified through inclusive composite interval mapping (ICIM) of the crosses, CSR28 x BRRI dhan28 and Hasawi x BRRI dhan28, respectively using ICIMapping software 4.2. Total size of the genetic linkage map for CSR28 x BRRI dhan28 was 1428.69 cM and it was 1556.57 cM for Hasawi x BRRI dhan28, constructed using 116 and 6209 SNP markers, respectively. The QTLs were identified in all the traits except in the productive tillers of the progenies from CSR28 x BRRI dhan28.

In the progenies of Hasawi x BRRI dhan28, most of the QTLs were found in productive tillers, panicle length, no filled spikelets and grain yield and most of them were located on chromosome 3 and 6. But no QTLs were found on chromosome 5 and 10 for this cross. In contrast, only chromosome 1, 3 and 10 had QTLs for different traits of the progenies from CSR28 x BRRI dhan28, but no QTLs was found in nine chromosomes (chromosomes 2, 4, 5, 6, 7, 8, 9, 11 and 12) in this cross.

Among the 15 QTLs detected in CSR28 x BRRI dhan28; two QTLs each were identified for plant height and percent filled spikelets, and one each for panicle length, number of filled and unfilled spikelets and grain yield. The largest outcome on phenotypic variation (14.5%) was identified on chromosome 10 for grain yield having a LOD value of 4.24. The tolerant parent, CSR28 contributed by additive effects to the QTLs for plant height, panicle length, number of filled and unfilled spikelets, percent filled spikelets and grain yield. But both the parents (BRRI dhan28 and CSR28) contributed alleles for plant height and percent filled spikelets.

In Hasawi x BRRI dhan28, eight QTLs for grain yield, seven QTLs each for panicle length and number of filled spikelets, six QTLs for productive tillers, three QTLs each for plant height and percent filled spikelets and a solo QTL for the number of unfilled spikelets were identified. The largest effect on phenotypic variation (27.03%) was identified on chromosome 1 for plant height with a LOD value of 14.87. Both tolerant (Hasawi) and sensitive (BRRI dhan28) parents contributed alleles to the QTLs for all traits except the plant height.

In both crosses, all the data were statistically confirmed with permutation analysis of 1000 times at 5% level of significance. Although more QTLs were identified in Hasawi x BRRI dhan28, but a few of them were statistically confirmed. Out of 48 QTLs in Hasawi x BRRI dhan28; two each for plant height, panicle length and percent filled spikelets, and three QTLs for productive tillers were statistically confirmed. In contrast, two QTLs for plant height and one each for number of filled spikelets, percent filled spiklets and grain yield were statistically confirmed for the progenies of CSR28 x BRRI dhan28.

This study suggested that genomic regions on chromosome 10 affects salinity tolerance at reproductive stage through an increase in number of filled spikelets, percent filled spikelet and grain yield of CSR28 x BRRI dhan28 progenies. These QTLs were mapped between the flanking markers, K_id10005402 and K_id100006100 at 75.93 cM (18.73-19.83 Mb). Candidate gene analysis indicated that a rice nuclear gene, *Rf-1* (chromosome 10 at 19.4 Mb) and basal transcriptional factor gene, *Osj10BTF3* (chromosome 10 at 18.69 Mb) was found near the identified QTLs in this study. And for Hasawi x BRRI dhan28, chromosome 3 affects salinity tolerance through increasing productive tillers, number of filled spikelets and grain yield, positioned between the flanking markers, CM020684.1_6731611_3 and CM020684.1_6810365 at 27.08 cM (~ 6.7 Mb). The identified QTLs were located near the sterile gene S34(t) mapped in between 2.43-10.0 Mb on chromosome 3. These loci are a good target for marker-assisted selection aimed at improving salinity tolerance. In addition, two QTLs for plant height for both crosses were located near the novel semidwarf (*Sd-1*) gene.

The QTLs identified in this study for reproductive-stage salt tolerance need to be fine mapped before they can be directly used to accelerate marker-assisted selection in future breeding programs to increase selection efficiency. The identification of the genes constituting these major QTLs would help to understand the molecular mechanisms.

Since rice plant is highly sensitive to the higher concentrations of sodium and its uptake causes Na^+ toxicity and osmotic stress; it is imperative to understand the molecular mechanisms of salt tolerance genes, particularly HKT genes to develop improved rice varieties for food security of the population living in the saline prone environment. Genome editing of HKT gene families with CRISPR/Cas9 enable efficient targeted modification in rice. This study targeted seven gene members of HKT gene family via multiplex CRISPR/Cas9 based DNA free genome editing which is time efficient. All the gRNAs were validated with *in vitro* RNP complex for future research work on gene editing of HKT gene family. Designing and pre-validating appropriate guide RNAs (gRNA) are indispensable for further steps like *in vivo* applications and gene delivery into a desired variety.