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Molecular Genetics of Cold Tolerance at Germination and Seedling Stages in Rice

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MOLECULAR GENETICS OF COLD TOLERANCE AT GERMINATION AND SEEDLING
STAGES IN RICE

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List of Abbreviations

BIL	backcross inbred lines
CIM	composite interval mapping
CIS-ACS	cold injury score after cold stress
CIS-ARP	cold injury score after recovery period in greenhouse
CL7d13C	coleoptile length after 7 days at 13°C
CL7d28C	coleoptile length after 7 days at 28°C
cm	centimeter
cM	centimorgan
CRI-CL	cold response index for coleoptile length
CRI-Germ	cold response index for germination
CRI-RL	cold response index for radicle length
CTG	cold tolerance at germination early
CTS	cold tolerance at seedling stage
ctw	hundredweight
Germ14d13C	germination% after 14 days at 13°C
Germ7d13C	germination% after 7 days at 13°C
Germ7d28C	germination% after 7 days at 28°C
IL	introgression line
LOD	logarithm of odds
Mb	mega base
Mha	million hectares
mL	milliliter

mm	millimeter
PCR	polymerase chain reaction
QTL	quantitative trait loci
RFLP	restriction fragment length polymorphism
RIL	recombinant inbred line
RL7d13C	radicle length after 7 days at 13°C
RL7d28C	radicle length after 7 days at 28°C
RtL-ARP	root length after recovery period in greenhouse
RtL-GH	root length in greenhouse experiment
SSR	simple sequence repeat
StL-ARP	shoot length after recovery period in greenhouse
StL-GH	shoot length in greenhouse experiment

Abstract

Low temperature stress is a major constraint for rice production in temperate and high altitude areas of the world. Delayed germination coupled with reduced seedling vigor hinders crop establishment and crop growth resulting in reduced rice productivity. Mapping of chromosomal regions controlling cold tolerance will accelerate marker-assisted breeding of cold tolerant rice varieties. A recombinant inbred line (RIL) mapping population involving a rice cultivar 'Bengal' and a US weedy rice accession 'PSRR-1' was evaluated for cold tolerance at both germination and seedling stages. Observations on germinating ability and seedling vigor were recorded under both low temperature (13°C) and optimum temperature (28°C) at germination stage. 'PSRR-1' performed better than 'Bengal' under cold stress. Forty-nine QTL distributed over ten chromosomes were identified for 11 traits. The number of QTL varied from one to nine with phenotypic variability of each QTL ranging from 3.5 to 12.7%. For 18 QTL, 'Bengal' alleles were adaptable, whereas 'PSRR-1' alleles improved germination and seedling vigor under cold stress in 31 QTL. Three major QTL were observed for coleoptile length and seedling shoot length. The QTL were clustered in six chromosomal regions. The congruency of a QTL cluster on chromosome 11 in this study with earlier studies suggests a potential target for cloning cold tolerance genes at germination stage. Evaluation of cold tolerance at seedling stage was conducted by exposing rice seedlings at the 3rd leaf stage to 18/8 ^{o}C day/night temperature for 18 days and scoring of visual damage on a 1-9 scale. 'Bengal' showed higher tolerance to cold stress than 'PSRR-1'. Twenty-three QTL for four cold tolerance traits were detected along all twelve chromosomes at seedling stage. The number of QTLs varied between four and eight per trait and each QTL contributed 2 to 42% of the total phenotypic variation. Bengal alleles increased cold stress tolerance in 10 QTL and PSRR-1 alleles in rest 13 QTLs. The most

prominent one was on chromosome 11 which harbored a cluster of cold tolerance QTLs at both

germination and seedling stage. This study demonstrated that weedy rice can be a valuable donor

for enhancing cold tolerance in rice cultivars.

Chapter 1. Introduction

1.1 Rice: Importance and Production

Rice (*Oryza sativa*) is one of the major food crops that provides about 20% of the daily calorie intake for more than half of the world's population. Its domestication from the wild rice species *Oryza rufipogon* traces back 8,200-13,500 years ago, to the Pearl River valley located in China (Maclean et al., 2013). Asia is the continent with the most rice producing countries, but it is also grown in other countries in Europe, Africa, Australia, and the American continent (FAO, 2010).

In the U.S., rice is predominantly grown in six states: Arkansas, California, Louisiana, Mississippi, Missouri, and Texas, with around 3 million acres of planted area. In 2009, rice production contributed more than $34 billion to the nation's economy and supported 128,224 jobs nationwide (Richardson & Outlaw, 2010). In Louisiana, rice has been cultivated since the early 17th century (LSU AgCenter, 2011) and is considered one of the most important crops of the state in terms of production and contribution to the state's economy. Rice farming in Louisiana contributed $2 billion to the state economy and supported 9,627 jobs (Richardson & Outlaw, 2010).

Considering the predicted increase of the world population, it is estimated that rice production should increase by 50% or more than the current levels to meet future food demand (Papademetriou, 2000). It seems to be a challenging task since rice production is facing many constraints, such as abiotic stresses, higher costs of production, and environmental issues. Abiotic stresses, particularly, represent a major threat for major rice producing countries (Mishra & Salokhe, 2008).

Rice breeding has been partly successful to overcome several major biotic and abiotic constraints. Its success in the latter half of the 20th century resulted in the development of semi-dwarf and early maturity varieties and subsequent improvement in resistance to diseases and insect pests, and cooking quality (Mackill, 2006). Despite the progress made in recent years, improving yield, nutritional quality, and resistance to abiotic stresses has been particularly challenging for rice breeders.

1.2 Rice Genetics, Ecology, and Biology

Rice (*Oryza sativa*) has the smallest genome compared to other cereal crops with a genome size of 430 Mb (Goff et al., 2002). Most of the *Oryza* species are diploid with 12 pairs of chromosomes (2n=24). A large number of *O. sativa* cultivars have been developed through centuries of rice domestication. They can be differentiated based on specific characteristics, such as adaptation to different water regimes, growth habit and height, shapes, size and color of the culm, leaf blade, panicle, hull and grain, degree of pubescence and tolerance to abiotic stresses (OECD, 1999). Two major subspecies of *O. sativa* have been identified: *indica* and *japonica*. Both are known to originate from the same domestication event but *japonica* subspecies is found in temperate regions whereas *indica* subspecies is grown predominantly in tropical and sub-tropical regions.

In most of the temperate rice-growing countries in Asia (Japan, Korea, and China), North America, Australia, and Europe, rice cropping is determined primarily by the temperature pattern. Rice is almost an entirely irrigated crop (De Datta, 1981). It thrives in a wide range of environments and under different climatic conditions. Due to this condition, most classifications are based on hydrological characteristics as follows: irrigated lowland, rainfed lowland, deep-water, and upland rice (Maclean et al., 2013). Irrigated lowland rice is grown in bunded fields

with ensured irrigation for one or more crops a year. Rainfed lowland rice is grown in bunded

fields that are flooded with rainwater for at least part of the cropping season to water depths that

exceed 100 cm for no more than 10 days. Deepwater rice and floating rice is found in flood-

prone environments, where the fields suffer periodically from excess water and uncontrolled,

deep flooding. Upland rice is grown under dryland conditions without irrigation and puddling,

usually in non-bunded fields. Current data shows that about 90% of worldwide rice production

comes from irrigated or rainfed lowland fields (Maclean et al., 2013). Rice can grow in different

types of soils, including saline and alkaline soils and chemical properties of the soil are not as

important as its physical ability to hold water (Scott et al., 2003).

Agronomically, the life cycle of rice has three different growth stages: 1) vegetative stage

begins with the germination period and finishes at the initiation of panicle primordia, 2)

reproductive stage begins from the initiation of panicle primordia to heading, and 3) ripening

stage is from heading to maturity. For purposes of this research, only germination and seedling

stage (vegetative growth) will be discussed.

1.2.1 Germination Stage

Germination is considered the initiation of the vegetative stage and starts after dormancy

is broken. It is defined as the stage in which the white tip of the coleoptile emerges from the seed

coat. There are many factors such as moisture content, temperature, varietal differences of

germination at low temperature and oxygen which influence germination in rice.

In terms of moisture content/water absorption, the germination process can be subdivided

into imbibition, activation, and post-germination growth stages. Rice germination studies

conducted by Takahashi (1961) showed that when seed is soaked it has 18 hours of water

absorption. This water intake is caused by imbibition forces that increase the seed's water

content to 25-35%. Even though the duration of this stage is known to be almost independent of temperature, water content will be influenced by it. On the other hand, in the activation stage, water content is low when compared to moisture levels in the imbibition stage. The duration of the activation stage is dependent on temperature. The activation stage is prolonged with lowering of the temperature. Once the activation stage is complete, germination stage begins with emergence of the coleoptile from the seed coat.

Temperature is an important factor at germination stage since it affects the duration of the activation stage and the growing process. Temperature effect during germination can be determined by three aspects: temperature, time, and germination percentage. Temperature ranging from 31-36°C allows germination to occur in about two days, however, in controlled conditions, when low temperature (between 0-5°C) are used for germination, the incubation period should be extended and sometimes it can take more than a month for a seed to germinate (Yoshida, 1981).

As mentioned before *O. sativa* is divided into two main cultivars, *indica* and *japonica*. Their geographical distribution suggests that *japonica* cultivars should have better performance when exposed to low temperature stress. Previous research carried out by Nagamatsu (1942) using *javanica, japonica,* and *indica* cultivars demonstrated that *javanica* and *japonica* cultivars germinated faster under low temperature compared to *indica* cultivars. Oxygen plays an important role in rice germination. Despite the fact that rice can germinate in adverse environment conditions, its growth might be severely stunted and abnormal when germinated in anaerobic conditions (Kordan, 1976).

1.2.2 Seedling Stage

The radicle and coleoptile continue to grow after germination until the development of leaves. When the tip of the first leaf has emerged, the plant is at the seedling stage. The rate of leaf development is highly affected by environment. Even for same locations and cultivars, variable weather conditions affect the rate of seedling growth. The following factors are considered to be of great importance during seedling development: temperature, light, oxygen, and water.

During the first week of post-germination, seedling development is highly dependent on temperature. Yoshida (1973), while evaluating the growth rate of the world's first high-yielding rice variety (IR8) at different temperature (22, 25, 28, and 31 °C), reported increase in growth rate with rise in temperature. At 22°C growth rate was significantly lower than growth rate at 25, 28, and 31°C, indicating that temperature ranging from 25-30°C is optimum for seedling development. Generally, critical temperatures may vary with variety, seed history, and cultural management. However, 10°C is considered as the critical minimum temperature for root and shoot elongation (Nishiyama, 1977). On the other hand, radicle growth is stopped at temperatures below 15°C and above 40°C (Yamakawa & Kishikawa, 1957). The elongation of the coleoptile, mesocotyl, first and second leaves, and internodes was faster with less exposure to light (Hoshikawa, 1975). Rice seed can germinate in anaerobic conditions. However, for seedling development, oxygen is essential for normal growth of the coleoptile, first leaf, and radicle. After the emergence of coleoptile, 5-6 ppm oxygen is required for seedling growth. With decline in oxygen level, root growth was inhibited but shoot growth increased (Tsuji, 1973; Alpi & Beevers, 1983).

1.3 Abiotic Stresses Affecting Rice Cultivation

Despite the ability of rice to grow in diverse environments, growth and development can be inhibited by several abiotic stresses such as drought, flooding, heat and cold; as well as Al and Fe toxicities, P and Zn deficiencies, and high soil salinity, (Martínez et al., 2014). The major rice producing countries including Japan, China, Korea, Brazil, India, the U.S., the Philippines, and Thailand are being affected by these abiotic stresses (Almeida et al., 2016). Of the 130 million ha of rice land, salinity and drought affect 30% and 20% of land, respectively where as 10% of land experience low temperature in high altitude areas of Japan (Shimono et al., 2007), Korea, China, Bangladesh, Nepal, India, and other countries (Lee, 2001; Kaneda & Beachell, 1974). In recent years, advances in physiology, molecular biology, and genetics have contributed to our understanding of how rice responds to these stresses and the basis of varietal differences in stress adaptation. The focus of this study was on cold stress in rice.

1.4 Cold Stress and its Negative Effects in Rice Cultivation

Cultivation of rice is negatively affected by cold stress in about 25 countries including the U.S. (Yoshida, 1981; Cruz et al., 2013). Cold temperature is distinguished from freezing by the range of temperatures that cause the injury. Cold stress injury can be categorized into two parts: (1) chilling injury which is caused by temperatures above freezing point (0-15°C), and (2) freezing injury, caused by temperature below freezing point (<0°C) (Roy & Basu, 2009). In general, plants from temperate regions exhibit more chilling tolerance, which can be increased by exposure to chilling or nonfreezing temperatures. This process is known as cold acclimation (Sanghera, 2011) which is associated with biochemical and physiological changes (Gilmour et al., 2000). Eventually changes in gene expression were displayed in biomembrane lipid composition and small molecule accumulation (Yamaguchi & Shinozaki 2006).

Several tropical and subtropical species are injured or killed by nonfreezing low temperatures. Numerous studies have been conducted to identify germplasm that perform better under exposure to low temperature. Overall, *japonica* cultivars are more tolerant than *indica* cultivars (Andaya & Mackill, 2003). According to Li et al. (1981), the cold stress damage in rice depends on the severity of the stress, the exposure duration, and the plant developmental stage. Satake and Hayase (1970) reported that low temperature stress in rice is more damaging at the booting stage. However, Yoshida (1981) found that cold susceptibility differs between stages. Based on his research, threshold temperature for cold injury in rice plants was 10-13°C during germination and vegetative stages, and 18-20°C during the reproductive stage. Recent studies suggest that rice growth and development is altered by cold stress at any developmental stage (Andaya & Mackill, 2003; Ye et al., 2009).

During early growth stages of rice, cold stress severely affects seed germination and subsequently, retards seedling establishment, eventually leading to non-uniform crop maturation. Rice plants are injured at the seedling stage when growing in early spring in temperate or subtropical environments. Temperatures below 15-17°C can cause severe injuries leading to poor establishment, decrease in the plant competitive ability against weeds, delayed crop maturation, and subsequently decrease in yield (Yoshida et al., 1996; Andaya & Makill, 2003; Farrell et al., 2006; Zhou et al., 2010). Poor germination, slow growth and discoloration of seedlings, stunted vegetative growth characterized by reduced height and tillering, delayed heading, incomplete panicle exertion, prolonged flowering period, degeneration of spikelets, irregular maturity, sterility, and formation of abnormal grains are typical symptoms caused by low temperatures (Kaneda & Beachell, 1974). Stunting was a common symptom at seedling stage and was highly correlated with weight growth of both shoot and root (Yoshida, 1981). He noticed that cool

weather conditions and cold reservoirs were responsible for stunting. In California, tolerance to cold water is important since rice seeds are sown in flooded areas with cold standing water. Delayed heading was observed in areas where summers are short and cool weather conditions are prolonged. Under these conditions, the rice crop may ripen under lower temperatures than usual and the grain filling process might not be completed (Tsunoda et al., 1966). Low temperature-induced sterility in rice is another important negative effect. Booting is considered the most sensitive stage to cold stress followed by heading or flowering (Satake & Hayase, 1970; Ye et al., 2009).

1.5 Methodologies to Evaluate Cold Stress in Rice

Evaluation of rice under cold stress conditions is important since it helps in the selection of cold tolerant germplasm for developing cold tolerant cultivars. Several methodologies have been used to evaluate rice under low temperature at different growth stages of the rice plant. Bertin et al. (1996) evaluated chilling sensitivity in different rice varieties at the germination stage. They screened rice seeds at 10, 15, 20 and 25°C for 3 to 30 days depending on the temperature and germination rates based on radicle protrusion were determined as indicators of cold tolerance. Similarly, Sthapit and Witcombe (1998) conducted experiments to determine the heritability of four traits: germination, the rate of germination index, plumule greening, and plumule vigor at 17°C for 7 days. They found that germination rate index and plumule greening ability were highly heritable traits and could be useful in breeding programs because of their heritability, relative ease to measure, and correlation with field performance at high altitudes. In Korea, Lee (2001) tested cold tolerance at the germination stage at 10, 12, and 15°C. His data showed that germination rates at 10°C were very low when compared with the rates at 12°C and 15°C, suggesting the influence of low temperature on lowering the germination rate. Hou et al.

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(2003) used coleoptile length to detect QTLs for low temperature (15°C for 10 days) germinability in rice. In another study, Chen et al. (2006) recorded germination rates at 15°C after 6 days of treatment. More recently, radicle length was adopted as a parameter to screen cold tolerance at different low temperature regimes (Bosetti et al., 2012; Pouramir-Dashtmian, 2013).

At the seedling stage, scientists have evaluated rice seedlings at the 2-leaf stage exposed at 10°C for 3, 6, and 9 days; survival rates after 10 days of recovery period is taken as indicator of cold tolerance (Bertin et al., 1996). Lee (2001) tested chilling injury in a cool-air treatment at 12°/10°C (day/night temperature) for 10 days at 3-leaf stage and considered growth and discoloration as parameters for screening cold tolerance. Another study used cold treatment as low as 4°C (constant temperature) for 6 days in the dark and then evaluated survival rate after 14 days of recovery (Koseki et al., 2010). Cold-tolerance was also evaluated by using the visual scale described in the Standard Evaluation Systems in rice (IRRI, 1988). Several studies have used that visual scale to evaluate rice seedlings at different temperature regimes (Andaya & Mackill, 2003; Andaya & Tai, 2006). Kim et al. (2014) conducted phenotypic evaluation of a recombinant inbred line (RIL) population at the 3 leaf-stage by subjecting to 18/8°C (day/night) for 18 days and then the cold injury score was recorded after 7 days of recovery period. Seedling survival percentage is the most common parameter to evaluate tolerance under cold stress in rice (Qian et al., 2000; Lou et al., 2007).

At the reproductive stage of rice, percentage of fertility is the most common parameter used to determine cold tolerance. It has been evaluated from the young microspore stage until the booting stage at different low temperatures ranging from 12 to 19.4°C (Koike et al., 1990; Cruz et al., 2006; Suh et al., 2010; Jena et al., 2012).

Physiological analyses can be done for cold tolerance screening. Most of the reported studies have been carried out at the seedling and booting stage. Chlorophyll content is used as criteria for evaluation since it has been demonstrated that it is highly reduced in cold susceptible cultivars (Dai et al., 1990; Aghaee et al., 2011). Tracking plant recovery after cold stress exposure has also been used (Kuk et al., 2003). In addition, membrane disruption is evaluated through electrolyte leakage analyses by taking total conductivity (Lee et al., 2004; Huang et al., 2009; Song et al., 2011; Zhang et al., 2011). When plants are exposed to stress, electrolytes are released through the cell membrane. The smaller the amount of electrolytes released, the higher the tolerance to the stress.

Rice growth under cold stress can be measured at germination, seedling, and reproductive stages. Taken all together, the methodologies described above have contributed to the understanding of rice plant response under low temperature stress. These methodologies have been useful for identification of cold tolerant rice cultivars.

1.6 QTL Mapping

A quantitative trait locus (QTL) is defined as 'the genetic region of any genome that is responsible for variation in a quantitative trait of interest' (Doerge, 2002). QTLs associated with cold tolerance have been identified through the use of molecular markers, which are DNA fragments that reveal genetic variation between individual organisms or species, and they have a specific position within the chromosomes (Collard et al., 2005). Molecular markers are useful in the construction of linkage map and QTL analysis. QTL mapping is a widely-used method that associates genotype with the quantitative trait of interest in a population exhibiting genetic variation for such trait.

Restriction fragment length polymorphism (RFLPs) and simple sequence repeat (SSR) markers have been used to construct linkage maps and to analyze QTLs that confer cold tolerance at different growth stages of the rice plant (Li et al., 1997; Yan et al., 1998; McCouch et al., 2002; Fujino et al., 2004; Suh et al., 2010). SSR markers are particularly valuable for the implementation of marker-assisted breeding strategies because they are co-dominant, detect high levels of allelic diversity, and are easily and economically tested by the polymerase chain reaction (PCR) (McCouch et al., 1997). Their genetic characteristics allow them to be efficiently used in both *indica* and *japonica* germplasm (McCouch et al., 2002).

In 2005, the International Rice Genome Sequencing Project (IRGSP) sequenced the rice genome which led to the development of high-quality and reliable single-nucleotide polymorphism (SNP) markers that are suitable for use in high-throughput technologies. SNP-based linkage maps have more power and resolution compared to those using RFLP and SSR markers (Mammadov et al., 2012). Few studies in rice involved SNPs for cold tolerance (Koseki et al., 2010). One study conducted at the biochemical level showed that differential response to cold stress in *indica* and *japonica* genotypes could be due to differences in enzymatic properties, specifically in the OsGSTZ2 isoform (Kim et al., 2011).

Cold tolerance studies carried out at different growth stages of the rice plant suggested that QTLs conferring cold tolerance are independent of growth stage. In the following two sections, previous studies regarding QTLs related to cold tolerance at the germination and seedling stages are discussed.

1.6.1 QTLs Related to Cold Tolerance at the Germination Stage of Rice

Optimum germination is critical to ensure fast and uniform crop establishment in direct seeded rice (Krishnasamy & Seshu, 1989). Several QTL analyses focused on germination under cold stress (Miura et al., 2001; Andaya & Mackill, 2003; Fujino et al., 2004; Chen et al., 2006; Fujino et al., 2008; Fujino & Matsuda, 2010; Ranawake et al., 2014). Those QTLs with the largest contribution were reported by Miura et al. (2001) and Fujino et al. (2004). Miura et al. (2001) used a set of 98 backcross inbred lines (BILs) from a cross between Nipponbare and Kasalath (*japonica* and *indica* cultivar, respectively) to identify five QTLs conferring cold tolerance at germination stage. These five QTLs were located on chromosome 2, 4, 5, and 11 that explained 41% of the total phenotypic variation. In case of three QTLs, Kasalath alleles increased cold tolerance whereas Nipponbare alleles were desirable for other two QTLs. In a mapping population consisting of 122 BILs derived from a cross between two *japonica* cultivars, Fujino et al. (2004) identified three QTLs on chromosomes 3 and 4 (*qLTG-3-1*, *qLTG-3-2,* and *qLTG-4*) with a total phenotypic variance of 58% for low temperature germinability. *qLTG-3-1* was a major QTL with 35% of the total phenotypic variation. Later, a map-based cloning revealed that *qLTG-3-1* is encoded by a protein of unknown function (Fujino et al., 2008). The authors suggested that this QTL may not be involved in the response to low temperature, but rather in seed germination.

In another study, while evaluating an F_2 population derived from an *indica/japonica* cross, eleven QTLs for low temperature germinability were detected on chromosomes 3, 4, 5, 7, 9, 10, and 11. For all QTLs, increased cold tolerance was due to *japonica* cultivar alleles (Jiang et al., 2006). Few other studies reported 2-5 QTLs responsible for low temperature tolerance at the germination stage (Zhang et al., 2005b; Chen et al., 2006; Ranawake et al., 2014).

In summary, most of the studies used plant material derived from *indica/japonica* crosses and backcross and F$_2$ populations were commonly used for cold tolerance at germination stage. Several QTLs were detected along the 12 chromosomes of rice, but there was only one major QTL affecting germination under cold stress.

1.6.2 QTLs Related to Cold Tolerance at the Seedling Stage of Rice

Tolerance to low temperature during seedling stage of rice plant is important to ensure stable early growth in temperate and high altitude areas. Consequently, development of cold tolerant cultivars at seedling stage is an important goal in rice breeding. Rice scientists screened several mapping populations to identify QTLs responsible for cold tolerance at this stage, and more than 80 QTLs were reported (Qian et al., 2000; Andaya & Mackill, 2003; Lou et al., 2007; Jiang et al., 2008; Baruah et al., 2009; Koseki et al., 2010; Jiang et al., 2011; Suh et al., 2012; Yang et al., 2013).

The *qCTS12* is a large effect QTL located on chromosome 12 that contributed over 40% of the phenotypic variance for cold-induced wilting tolerance and cold-induced necrosis tolerance in a RIL population (Andaya & Tai, 2006). In another study, using a double haploid mapping population, five QTLs were identified on chromosome 1, 2, and 8. *qCTS-2* was a major QTL on chromosome 2 that explained 27% of total phenotypic variation for cold tolerance at seedling stage (Lou et al., 2007). Same QTL was also reported by Liu et al. (2013) in an F$_{2:3}$ population.

Major QTLs related to cold tolerance at seedling stage were also reported in other chromosomes (Zhang et al., 2005; Lou et al., 2007; Park et al., 2013; Zhang et al., 2014). A prominent one was found in chromosome 4 (*qSPA-4*) in a study that used leaf discoloration as indicator of cold sensitivity. It accounted for 16% of the phenotypic variation in a RIL and an

introgression lines (ILs) population derived from an intersubspecific cross involving weedy rice (Park et al., 2013).

The above described studies used populations largely derived from *indica/japonica* crosses with the exception of Park et al. (2013).

1.7 Weedy rice and its importance in rice breeding

Weedy rice (*Oryza sativa* f. *spontanea*) is a rice-like weed distributed all over the world in rice growing regions. It is known to have genetics, physiology, and life history similar to that of cultivated rice (Cao et al., 2007). Shattering ability and dormancy are intrinsic characteristics of weedy rice that contribute to its easy dissemination. Due to its red pericarp color, weedy rice is commonly referred as red rice. As many other weeds, it competes for nutrients, water, sunlight, and other resources with cultivated rice, and consequently becomes a challenge in crop management (Dekker, 1997). Although it has many undesirable attributes, its genetic flexibility and phenotypic plasticity provides a great potential for adaptation to a wide range of agro-ecosystems, including abiotic stresses conditions. Through years of breeding efforts, rice breeders have discovered that genes present in wild and weedy relatives of rice can enhance the performance of some of the world's most productive crop varieties (McCouch, 2004). The exploration of the weedy rice gene pool could lead to the finding of beneficial genetic resources for rice breeding.

1.8 Breeding for Cold Tolerance in Rice

The development of cold tolerant rice varieties is the most effective method to mitigate cold stress injury. Genetic variation is required for improving trait performance through breeding. Research on breeding cold-tolerant varieties and cultural practices began in 1970 (Lee, 2001). Since then, it continues to be an important breeding objective because *indica/japonica*

hybrids and high-yielding rice cultivars were found to be highly susceptible to low temperatures. The existence of cold-tolerant germplasm facilitates the breeding for cold tolerance. *Indica* and *japonica* cultivars have been investigated extensively to evaluate varietal differences that will favor the selection for cold tolerance. *Japonica* cultivars were more cold-tolerant than *indica* cultivars. This finding was supported by Lee (2001), who reported that *japonica* cultivars had germination rates significantly higher than those of *indica* cultivars under controlled conditions (10, 12, and 15°C). Also, Mertz et al. (2009) evaluated physiological changes in rice seeds exposed to cold in the germination stage in four rice cultivars (two *indica* cultivars and two *japonica* cultivars) and noted that germination percentage, germination speed, coleoptile length, and dry weight at 13ºC were higher in *japonica* genotypes than in *indica* genotypes. Likewise, Li et al. (1981) found correlation of cold tolerance at different growth stages in rice and *japonica* cultivars performed better than *javanica, indica,* and wild rice. Mackill and Lei (1997) reported that temperate *japonica* cultivars have better seedling-stage cold tolerance than *indica* cultivars, which showed higher chlorophyll content, higher seedling vigor, shorter growth duration, higher panicle exertion, and lower shattering when evaluated at 9°C and 13°C. Another research study concluded that there is variability of cold tolerance level at germination stage within subspecies (Cruz & Milach, 2004). The *indica* cultivars from high altitude areas had moderate level of cold tolerance (Jennings et al., 1979). Ikehashi (1973) compared four *japonica* cultivars and four *indica* cultivars, and concluded that *indica* cultivars had higher germination percentage in less time than *japonica* under low temperature (9-11ºC).

Selection for cold tolerance in field conditions is challenging due to the unpredictability of intensity, duration, and timing (Cruz & Milach, 2000). For this reason, it is crucial to have a reliable selection method to evaluate cold tolerance by using controlled air and water

temperature. Several methodologies have been described to screen rice genotypes under low temperature conditions (Cruz et al., 2013). The advantage of evaluating rice under controlled conditions is that duration and precision of the stress can be manipulated. However, available space becomes a problem when large populations are to be evaluated.

In addition to a reliable selection method, the genetic basis of cold tolerance is important for improving this trait. Cruz et al. (2006) determined inheritance and heritability of cold tolerance at the germination stage in crosses between six rice genotypes. They concluded that both additive and non-additive gene action were involved but the non-additive action was relatively more important for percentage reduction in coleoptile length indicating complex genetics associated with cold tolerance at the germination stage in rice. At the seedling stage, cold tolerance was reported to be controlled by a single dominant gene (Shahi & Khush, 1986; Xu & Shen, 1988). Although this was supported by Nagamine (1991), he found no relationship between chilling injury and low temperature chlorosis indicating control of each trait by different locus. Two major genes were responsible for cold tolerance at the vegetative stage; *Cts1* and *Cts2*, responsible for leaf yellowing and leaf withering, respectively (Kwak et al., 1984; Nagamine, 1991). Despite the fact that major genes have been identified for seedling cold tolerance, a recent QTL analysis suggested that cold tolerance is a complex trait involving multiple genes (Andaya & Tai, 2006). For instance, at the reproductive stage, cold tolerance was reported as a polygenic trait (Acharya and Sharma, 1983; Khan et al., 1986; Kaw et al., 1989). Even though breeding for cold tolerance is challenging due to complex inheritance and some other limitations, many breeders consider selection for tolerance in early generations is effective (Toriyama & Futsuhara, 1960; Futsuhara & Toriyama, 1969,1971; Khan et al., 1986).

1.9 Rationale for Research and Objectives

Quantitative trait loci (QTL) mapping is an important tool to identify chromosomal regions controlling complex traits. Marker-assisted selection is gradually becoming an effective tool to improve quantitative traits that are controlled by a few QTLs with large effects. QTLs have been identified for tolerance to abiotic stresses, such as, salinity (Thomson et al., 2010; Ammar et al., 2011), low temperature stress (Andaya et al., 2003; Fujino et al., 2004 Chen et al., 2006) and, Fe and Al toxicity (Nguyen et al., 2003; Wan et al., 2003; Wu et al., 2014). Some advances have been made in improving tolerance to drought stress (Courtois et al., 2000). Temperature stress studies often focused on low temperature stress as it is a common limiting factor in both temperate and subtropical areas and high altitude areas. Rice is more sensitive to cold stress than other cereal crops such as wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Cruz & Milach, 2004). In temperate regions, rice production is negatively impacted by cold stress (Xie et al., 2012). Since rice can be grown in diverse locations and climatic conditions, specific criteria for evaluation and selection of cold tolerant lines have been established at specific growth stages. Commonly, evaluation of rice populations is done during the seedling and reproductive stage, which are critical to rice production. However, in regions with long, cold springs, germination can be inhibited and consequently affects early seedling growth. For this reason, evaluation of cold tolerance at the germination stage is also important in those regions.

Since rice growing areas in the southern USA experience low temperature stress during germination and seedling stage resulting in delayed germination and uneven stand in field, this study was carried out to identify QTLs for tolerance to low temperature stress at both germination and seedling stage using a recombinant inbred line (RIL) developed from a cross

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between Bengal and a weedy rice accession PSRR-1. The overall goal of this study was to investigate the genetics of cold tolerance at both germination and seedling stages of rice and demonstrate the utility of weedy rice for rice improvement.

The specific objectives of this study were:

1. To identify QTLs for cold tolerance at the germination stage using a RIL population developed from the Bengal x PSRR-1 cross.

2. To identify QTLs for cold tolerance at the seedling stage using the same RIL population.

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Chapter 2. Genetic Analysis of Germinating Ability and Seedling Vigor under Cold Stress in US Weedy Rice[*]

2.1 Introduction

Rice is cultivated all over the world with a wide range of variation in geography. Compared with the tropical and subtropical regions, rice grown in temperate and high altitude regions is frequently exposed to low temperature during the growing season. Cold stress is a major constraint to rice cultivation in 25 countries, including the United States (Yoshida, 1981; Pereira da Cruz et al., 2013). Although rice is sensitive to cold stress, a wide range of genetic variation for cold tolerance exists in rice germplasm (Baruah et al., 2009; Bosetti et al., 2012) and *indica* cultivars are less tolerant than the *japonica* cultivars (Andaya & Mackill, 2003).

In direct seeded rice, germination ability, early seedling emergence, and seedling vigor are crucial for rapid and uniform crop establishment, and increased ability to compete with weeds in rainfed and upland rice cropping system. Staggered germination results in variation in maturity resulting in rice crop with poor quality. Fast germination and seedling growth under low temperature is a major breeding objective particularly in direct seeding cropping system in rice growing areas that experience low temperature stress.

Rice plants are damaged by exposure to cold stress during all stages of plant growth. The optimal temperature range for germination and seedling establishment is 25ºC to 35ºC (Chapman & Peterson, 1962). However, a number of factors such as varietal difference, duration of cold stress, and physiological stage of the plant are responsible for variation in cold tolerance of rice (Yoshida, 1981; Nishiyama, 1985). In rice breeding, cold tolerance is usually evaluated at germination, seedling, and reproductive stages. Since field evaluation is challenging, methodologies to evaluate cold tolerance in controlled condition have been developed. Jones and

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Peterson (1976) developed a slant board test procedure to evaluate seedling vigor under cold stress and noted that shoot length of 15-d old seedling at 18°C is significantly correlated with seedling vigor under field conditions. Correlation between coleoptile length and seedling establishment under low temperature in field condition has been reported (Ogiwara & Terashima, 2001). The evaluation of germination index, seedling survival percentage, coleoptile length, radicle length, seedling length, and root length under low temperature has been a common practice in a wide range of studies such as germplasm and cold tolerant near-isogenic lines characterization (Cruz & Milach, 2004; Sharifi, 2010; Zhou et al., 2012), inheritance studies (Sthapit & Witcombe, 1998), and QTL mapping (Fujino et al., 2004).

A number of QTL mapping studies focused on germination under cold stress (Miura et al., 2001; Fujino et al., 2004; Liang et al., 2006; Ranawake et al., 2014) and early seedling cold tolerance (Zhang et al., 2005a; Lou et al., 2007; Liu et al., 2015). In Nipponbare x Kasalath cross, Miura et al. (2001) identified five QTL for germination under low temperature stress on chromosomes 2, 4, 5, and 11 with a total phenotypic variance of 41%. Nipponbare alleles were beneficial in case of two QTL while Kasalath improved germination under cold stress in the rest. Liang et al. (2006) used an *indica* x *japonica* cross and identified two QTL for low temperature germination ability on chromosomes 3 and 10. Fujino et al. (2004) identified three QTL on chromosomes 3 and 4 for low temperature germination ability in a cross involving temperate *japonica* varieties. Of these, the major QTL *qLTG3-1* encoding a protein of unknown function was cloned (Fujino et al., 2008).

Ranawake et al. (2014) studied cold tolerance at germination (CTG) and early seedling (CTS) stages in a *japonica* x *indica* cross. The cold tolerance QTL in their study were dependent on growth stages. Using seedling survival percentage after cold stress as a criterion, multiple

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QTL were mapped in several studies (Zhang et al., 2005a; Lou et al., 2007; Liu et al., 2015). In another study, Zhang et al. (2005a) identified 34 QTL for four seedling vigor traits (germination rate, root length, shoot length, and dry weight) at three temperature regimes (25°C, 20°C, and 15°C). Most of these were concentrated in five chromosomal regions with individual QTL contribution ranging from 3-16% of the phenotypic variance. Significant genotype x temperature regime interaction was observed. Among the four seedling vigor traits, shoot length and germination rate were concluded as good predictor for seedling vigor in rice. Combining QTL mapping with micro-array data, Liu et al. (2013) reported a candidate gene (LOC_Os07g22494) that improved cold tolerance at early seedling stage.

Only one QTL mapping study involved a RIL population involving a *japonica* weedy rice, which was evaluated for cold tolerance at the reproductive stage (Oh et al., 2004). This study demonstrated the utility of weedy rice as potential donor in rice breeding. Although weedy rice belongs to the same genus and species as cultivated rice (Hoagland & Paul, 1978), it is noted for its persistence and survival due to its unusual genetic variability and phenotypic plasticity (Oka, 1988). The weedy rice infestation is a major challenge for the rice farmers in southern United States and many parts of the world (Webster, 2000). Molecular marker-based investigation showed that weedy rices are closer to *indica* or *japonica* subspecies of rice rather than the wild species *Oryza rufipogon* (Suh et al., 1997). But weedy rice accessions of the United States were genetically more diverse and some accessions were found to be closer to *O. nivara* or *O. rufipogon* (Vaughan et al., 2001). Weedy rice has many desirable attributes such as rapid seedling growth, higher root mass, deeper root, seedling resistance to pathogen attack (Lee et al., 2000) and tolerance to various biotic and abiotic stresses (Suh et al., 1997, 1999), which can be exploited to boost rice productivity. Unlike wild species of rice, weedy rice hybridizes easily

making fertile hybrids and facilitates development of populations for investigating the genetics of useful agronomic and domestication traits.

In this study, we demonstrated that the US weedy rice has enhanced seedling vigor even under cold stress and further identified QTL for cold tolerance and seedling vigor based on seedling attributes in a mapping population involving a US weedy rice accession. Our results suggest that US weedy rice can be an invaluable resource for rice breeding programs to improve both germination and seedling vigor under cold stress.

2.2 Materials and Methods

2.2.1 Plant Materials

A population of 198 RILs in the $F_{7:8}$ generation was developed from the cross between a medium grain high yielding rice cultivar 'Bengal' (Linscombe et al., 1993) and a weedy rice accession PSRR-1 (Subudhi et al., 2012). PSRR-1 was collected from the Rice Research Station at Crowley, LA and was purified by single plant selection for two generations before crossing to develop the mapping population. It has light green leaves, vigorous growth, long auricles and ligules, straw-hulled medium grains, lax open panicles, and pubescent leaves. This weedy rice accession is extremely susceptible to shattering and has a higher intensity of both hull and pericarp dormancy compared to Bengal.

2.2.2 Evaluation of Cold Tolerance

For each RIL and parents, seeds were treated at 50°C for 5 days to eliminate residual dormancy. Before initiating the experiment, 8 randomly selected RILs and parents were tested for germination. After the germination was tested, seeds of the RIL population and parents were placed in petri dishes lined with a layer of germination paper (Anchor Paper Co.) with addition of 10 mL of distilled water. The petri dishes were then placed in a basin covered with Saran®

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wrap and transferred to an incubator set at 28°C for pregermination in darkness. After 2 days of incubation at 28°C, the pregerminated seeds were transferred to incubators for germination in dark condition under optimum temperature (28°C) or cold stress (13°C) for 7 and 14 days, respectively.

A randomized block design with three replications was followed for cold tolerance evaluation. For each line, 20 seeds were used for germination in each replication. Besides germination percentage, observations were recorded on 5 randomly selected germinating seeds for coleoptile length (CL) and radicle length (RL). For normal condition, the measurements were taken only on the 7th day after incubation. But for cold stress, the measurements were recorded on the 7th day and 14th day after incubation. Splitting of the hull by the emerging radicle was used as the criterion for visible germination. The number of seeds germinated in each replication of each RIL and parent was expressed as a percentage. The percent germination was arcsine transformed to improve the normality of distribution for statistical analysis and QTL mapping.

To evaluate the response of parents and individual RIL, cold response index (CRI) was calculated using the formula [CRI = (Phenotypic value after 7 days of incubation at 13°C) / (Phenotypic value after 7 days of incubation at 28°C) ×100]. The degree of susceptibility to cold stress at germination stage was ascertained from the CRI values.

All traits were abbreviated as follows: germination percent after 7 days of incubation at 28°C (Germ7d28C), 13°C (Germ7d13C), and 14 days of incubation at 13°C (Germ14d13C); coleoptile length after 7 days of incubation at 28°C (CL7d28C), 13°C (CL7d13C), and 14 days of incubation at 13°C (CL14d13C); radicle length after 7 days of incubation at 28°C (RL7d28C), 13°C (RL7d13C) and 14 days of incubation at 13°C (RL14d13C); CRI for germination (CRI-Germ), CRI for coleoptile length (CRI-CL), CRI for radicle length (CRI-RL).

After evaluating cold tolerance in the laboratory, the germinated seeds were transferred to the greenhouse with 26ºC/18ºC day/night temperature in order to assess the development of the plants in soil. The experiment was conducted in a randomized block design with three replicates. Ten germinated seeds per each RIL were sown in a tray in each replication and 5 random plants per line were measured for shoot length (StL-GH) and root length (RtL-GH) at14 days after planting in the greenhouse.

2.2.3 Statistical Analyses

Analysis of variance (ANOVA) and mean comparison were done using the GLIMMIX procedure. Lines were entered as the fixed effect and the replications were treated as random effect. To improve the normality, data were transformed prior to analysis. Percent germination data were arcsine transformed while the coleoptile and radicle length data were transformed by taking the square root of the value anchored to 0.5. Pearson correlation coefficients among traits were computed based on RIL means using the CORR procedure. Heritability of each trait was estimated following Holland et al. (2003). All analyses were carried out using SAS (SAS Institute 2011). All histograms were made in Microsoft Excel 2010.

2.2.4 QTL Analysis

A linkage map developed in this RIL population (Subudhi et al., 2012) was used for QTL mapping. The linkage map consisted of 212 simple sequence repeat (SSR) markers and a morphological marker *Rc* (the pericarp color) with a total map distance of 1410 cM and average marker interval of 6.6 cM. Both single marker analysis and composite interval mapping (CIM) were performed using QTL Cartographer version 2.5 (Wang et al., 2011). In the CIM procedure, a forward-backward regression procedure with 20 cofactors was followed with walk in speed of 1.0 cM for detection of QTL. Logarithm of odds (LOD) score of 2.5 was used as the threshold

for declaring significance of the QTL. Since QTL identified using data taken after 14 days of exposure at 13°C were same as those from 7 days at 13°C, those data were not provided. The QTL were named following McCouch et al. (1997). For example, the QTL located on chromosome 3 for germination percentage after 7 days of exposure to 28°C was named as *qGerm7d28C-3*.

2.3 Results

2.3.1 Variation in early seedling vigor and cold tolerance response among parents and RILs

Analysis of variance revealed significant differences with respect to all traits in the RIL population except RL7d28C. Descriptive statistics with respect to germination and early seedling vigor traits under both normal and cold stress were presented in Table 1. Bengal and PSRR-1 did not differ in the germination rate 7 days after incubation at 28°C, but there were significant differences in coleoptile length and radicle length (Fig. 1). For both traits, weedy rice accession PSRR-1 showed better performance compared with Bengal.

Low temperature stress reduced germination percentage significantly in 'Bengal' after 7 and 14 days. However, PSRR-1 maintained the same germination rate under cold stress as under optimum condition. At both time points, germination remained around 70% in Bengal. Cold stress seriously damaged the coleoptile and radicle growth. But PSRR-1 grew significantly faster compared with Bengal. To compare the performance of both parents under cold stress relative to optimum condition, cold response index was calculated. For all three traits, PSRR-1 was better than Bengal. To observe the impact of cold stress at early seedling stage, seedlings were planted

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Table 1. Descriptive statistics for cold tolerance-related traits during germination and early seedling stage in Bengal x PSRR-1 RIL mapping population of rice.

Trait	Bengal Mean	PSRR-1 Mean[#]	BR-RILs Mean	BR-RILs Range	Pr > F[$]	Heritability
Germ7d28C (%)	99.99	99.99[ns]	75.55±0.86	19.50-89.36	<.0001	0.92
Germ7d13C (%)	86.67	99.99[**]	59.81±1.40	4.73-89.36	<.0001	0.67
Germ14d13C (%)	88.33	99.99[**]	70.28±1.12	4.73-89.36	<.0001	0.87
CL7d28C (mm)	48.33	73.00[**]	39.83±0.64	4.20-70.20	<.0001	0.80
CL7d13C (mm)	0.47	2.87[*]	3.02±0.10	0.07-7.40	<.0001	0.20
RL7d28C (mm)	71.33	92.00[ns]	43.62±0.91	3.20-78.13	0.0014	0.38
RL7d13C (mm)	0.60	4.40[*]	2.96±0.13	0.00-10.53	<.0001	0.17
CRI (Germ)	77.06	100.00[**]	77.74±1.49	9.62-126.68	<.0001	0.79
CRI (CL)	0.97	3.93[ns]	7.54±0.25	0.69-22.42	<.0001	0.80
CRI (RL)	0.84	4.78[*]	7.24±0.39	0.02-30.99	0.0105	0.79
StL-GH (cm)	27.93	31.33[*]	23.04±0.39	4.80-34.67	<.0001	0.40
RtL-GH (cm)	6.47	5.31[ns]	7.79±0.13	2.20-12.07	<.0001	0.29

Germ7d28C, arcsine transformed values of germination% after 7 days at 28°C; Germ7d13C, arcsine transformed values of germination percentage after 7 days at 13°C; Germ14d13C, arcsine transformed values of germination% after 14 days at 13°C; CL7d28C, coleoptile length after 7 days at 28°C; CL7d13C, coleoptile length after 7 days at 13°C; RL7d28C, radicle length after 7 days at 28°C; RL7d13C, radicle length after 7 days at 13°C; CRI-Germ, cold response index for germination; CRI-CL, cold response index for coleoptile length; CRI-RL, cold response index for radicle length; StL-GH, shoot length in greenhouse experiment; RtL-GH, root length in greenhouse experiment.
[#] Differences in trait means between Bengal and PSRR-1.
ns, not significant * significant at P<0.05, ** significant at P<0.01.
[$] Significant difference among the RILs of the Bengal x PSRR-1 mapping population based on F-test from the ANOVA.
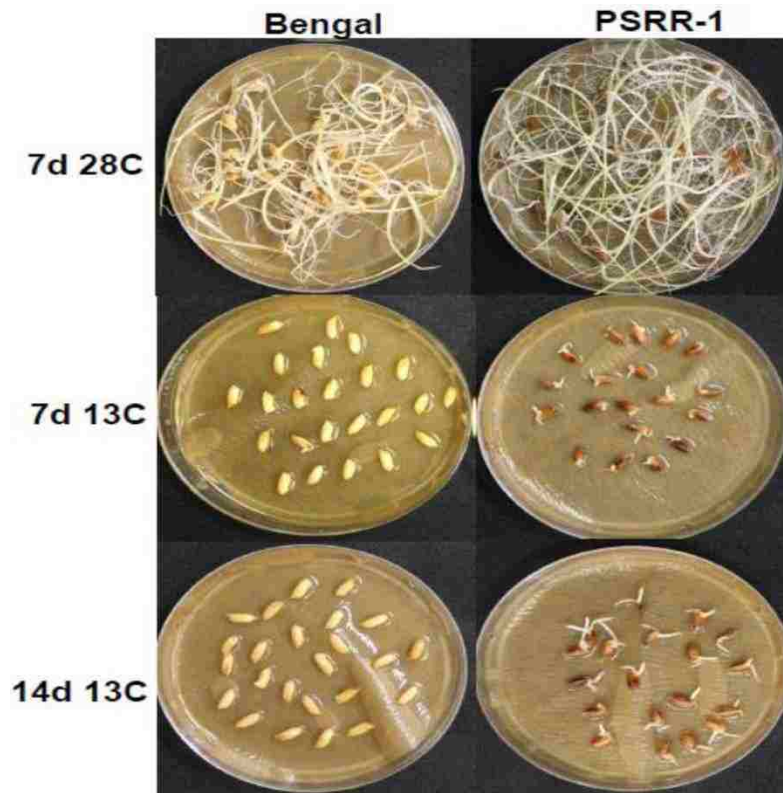
Figure 1. Germination of parents under optimum temperature and cold stress. Pictures were taken 7days after incubation at 28°C (Top) and 13°C (middle), and 14 days after incubation at 13°C (bottom).

in the greenhouse under normal temperature for 14 days. PSRR-1 had significantly greater shoot length compared to Bengal, whereas there was no difference in root length.

There was a wide range of variability in the population. For the traits, Germ7d28C, Germ7d13C, and Germ14d13C, distribution was skewed (Fig. 2). The distribution for coleoptile and radicle length at both optimum and low temperatures was normal (Fig. 3). The distribution for CRI (CL) was normal but the CRI for other two traits were a little skewed (Fig. 4). The traits

Figure 2. Frequency distribution of germination% under cold stress in the RIL population of the cross Bengal x PSRR-1. Mean values for parents and RIL population are marked by arrows. B, P, and R represent for Bengal, PSRR-1, and RIL population, respectively. Germ7d28C, Arcsine transformed values of germination% after 7 days at 28°C; Germ7d13C, Arcsine transformed values of germination% after 7 days at 13°C; Germ14d13C, Arcsine transformed values of germination% after 14 days at 13°C.
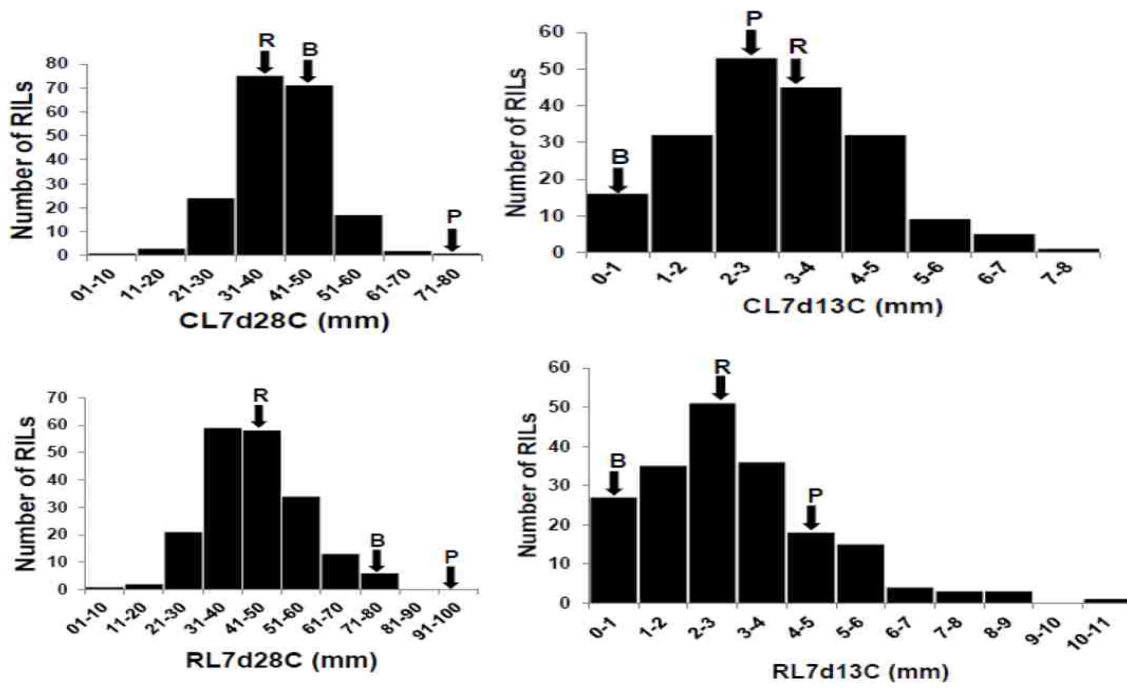
Figure 3. Frequency distribution of coleoptile length and radicle length under cold stress in the RIL population of the cross Bengal x PSRR-1. Parental means are marked by arrows. B, P, and R represent mean values for Bengal, PSRR-1, and RIL population, respectively. CL7d28C, Coleoptile length after 7 days at 28°C; RL7d28C, Radicle length after 7 days at 28°C; CL7d13C, Coleoptile length after 7 days at 13°C; RL7d13C, Radicle length after 7 days at 13°C; CL14d13C, Coleoptile length after 14 days at 13°C; RL14d13C, Radicle length after 14 days at 13°C.
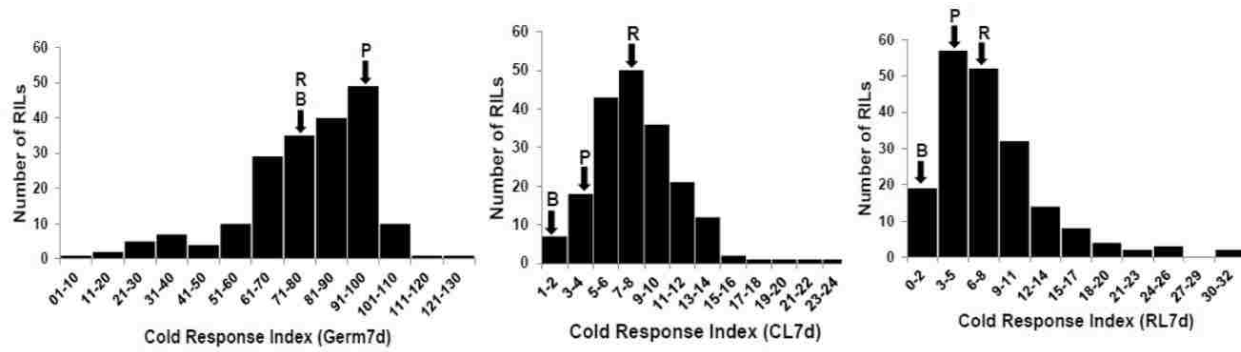
Figure 4. Frequency distribution of cold response indices in RIL population of the cross Bengal x PSRR-1. Mean values for parents and RIL population are marked by arrows. B, P, and R represent for Bengal, PSRR-1, and RIL population, respectively. Cold response indices were calculated as [(trait mean at 13°C/trait mean at 28°C) x 100].

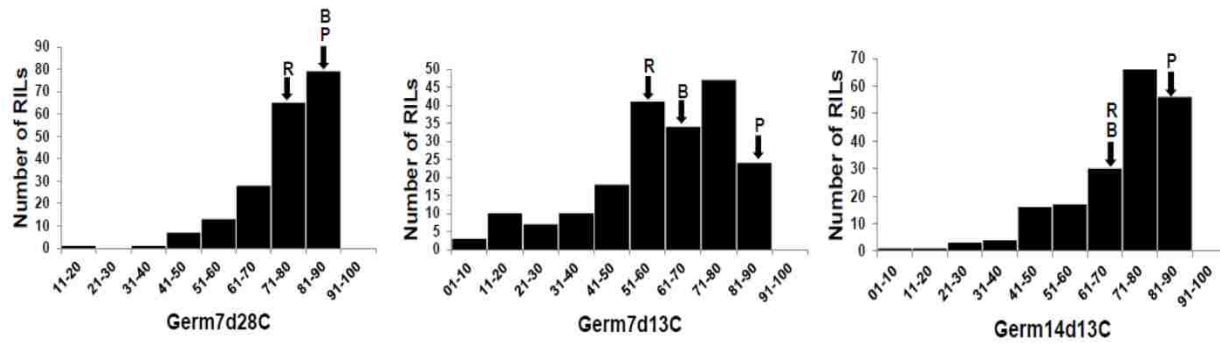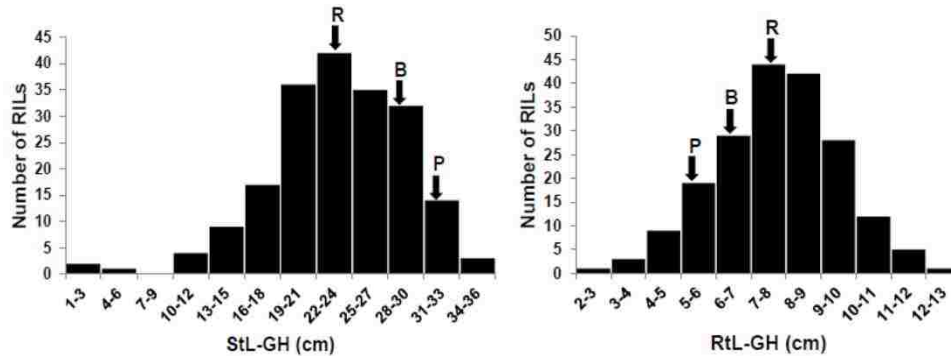**Figure 5.** Frequency distribution of shoot length and root length 14 days after transplanting in greenhouse in RIL population of the cross Bengal x PSRR-1. The germinated seeds were transferred to greenhouse after the cold tolerance evaluation in the laboratory. Mean values for parents and RIL population are marked by arrows. B, P, and R represent for Bengal, PSRR-1, and RIL population, respectively. StL-GH, shoot length in greenhouse experiment; RtL-GH, root length in greenhouse experiment.

StL-GH and RtL-GH were normally distributed with many of the RILs falling outside of the

parental range indicating transgressive segregation (Fig. 5). Mean values of the RIL population

was lower than both parents for Germ7d28C, Germ7d13C, CL7d28C, and RL7d28C but higher

than the parent with higher mean for CL7d13C, CRI (CL), and CRI (RL) and similar or within

the parental range in the rest.

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Broad sense heritability was moderate to high for Germ7d28C, Germ7d13C, Germ14d13C, CL7d28C, CRI (Germ), CRI (CL), and RL7d13C, and was low for CL7d13C, RL7d28C, CRI (RL), StL-GH, and RtL-GH.

2.3.2 Correlations among traits

As shown in Table 2, the phenotypic correlations among all germination and early vigor traits were all positive and highly significant (P <0.001). The only negative and significant correlation was observed between CRI-RL and RL7d28C. The germination percentage under optimum temperature was highly correlated to traits measured under both optimum and low temperature stress, but the radicle length under optimum condition or root length under greenhouse was either not correlated with many of these traits or the strength of most correlations was low. The cold response index for coleoptile length and radicle length was not correlated with the seedling traits measured in greenhouse after exposure to cold stress.

2.3.3 Quantitative trait loci for germination ability and seedling growth under cold stress

Scanning of the whole genome detected 49 QTL for eleven traits distributed along ten chromosomes under both control and low temperature stress environments (Table 3 and Fig. 6). Thirty QTL were clustered within six regions of five chromosomes 1, 3, 8, 11, and 12. The number of QTL varied between one and nine per trait and each QTL contributed 3.5 to 12.7% of the total phenotypic variation. The weedy PSRR-1 alleles increased the trait means in 31 QTL and Bengal alleles were favorable in the rest.

Further examination of QTL results revealed the following points. Only one QTL was detected for Germ7d28C with Bengal allele improving germination percentage, but three QTL were detected under cold stress including the one for Germ7d28C. For the two QTL, PSRR-1

allele improved germination under cold stress. For the cold response index, two of the four QTL were same as the QTL for Germ7d13C and two were new.

For traits, CL7d28C, CL7d13C, and CRI-CL, 6, 8, and 9 QTL were detected (Table 3; Fig. 6). Only one QTL on chromosome 2 was common under both optimum and cold temperature. Five QTL located on chromosomes 1, 3, 8, and 12 were identical with respect to the direction of additive effect between CRI-CL and CL7d13C. Only one major QTL from PSRR-1 accounting for a phenotypic variation of 11% was located on chromosome 8. For RL7d28C and RL7d13C, three QTL were detected for each trait, but six QTL were identified for CRI-RL. In case of two of the 3 QTL for CL7d28C, Bengal allele was desirable. There were two common QTL on identical position for these traits. None of these QTL explained phenotypic variation greater than 10%. There were three QTL each identified for StL-GH and RtL-GH and PSRR-1 contributed the allele for cold tolerance in case of two QTL. There was no overlapping of QTL between them. But there was one major QTL on chromosome 11 explaining 11% of phenotypic variation and desirable allele was from PSRR-1.

Table 2. Pearson correlation coefficients between the early seedling stage cold tolerance and seedling vigor traits in the RIL mapping population of rice cross Bengal x PSRR-1.

	Germ 7d28C	Germ 7d13C	CL 7d28C	RL 7d28C	CL 7d13C	RL 7d13C	CRI-Germ	CRI-CL	CRI-RL	StL-GH	RtL-GH
Germ7d28C	1.000										
Germ7d13C	0.739***	1.000									
CL7d28C	0.565***	0.602***	1.000								
RL7d28C	0.396***	0.325***	0.390***	1.000							
CL7d13C	0.450***	0.715***	0.469***	0.157*	1.000						
RL7d13C	0.426***	0.645***	0.341***	0.019	0.750***	1.000					
CRI-Germ	0.436***	0.912***	0.506***	0.225**	0.697***	0.605***	1.000				
CRI-CL	0.288***	0.548***	0.058	0.009	0.883***	0.669***	0.581***	1.000			
CRI-RL	0.289***	0.491***	0.177*	-0.298***	0.637***	0.907***	0.489***	0.648***	1.000		
StL-GH	0.351***	0.510***	0.387***	0.323***	0.299***	0.218**	0.479***	0.179*	0.089	1.000	
RtL-GH	0.243***	0.281***	0.198**	0.157*	0.141	0.150*	0.219**	0.073	0.099	0.338***	1.000

Germ7d28C, arcsine transformed values of germination% after 7 days at 28°C; Germ7d13C, arcsine transformed values of germination percentage after 7 days at 13°C; Germ14d13C, arcsine transformed values of germination% after 14 days at 13°C; CL7d28C, coleoptile length after 7 days at 28°C; CL7d13C, coleoptile length after 7 days at 13°C; RL7d28C, radicle length after 7 days at 28°C; RL7d13C, radicle length after 7 days at 13°C; CRI-Germ, cold response index for germination; CRI-CL, cold response index for coleoptile length; CRI-RL, cold response index for radicle length; StL-GH, shoot length in greenhouse experiment; RtL-GH, root length in greenhouse experiment.
Significance of correlation coefficients were indicated by * (P-values <0.05), ** (P-values <0.01), and *** (P-values<0.001).
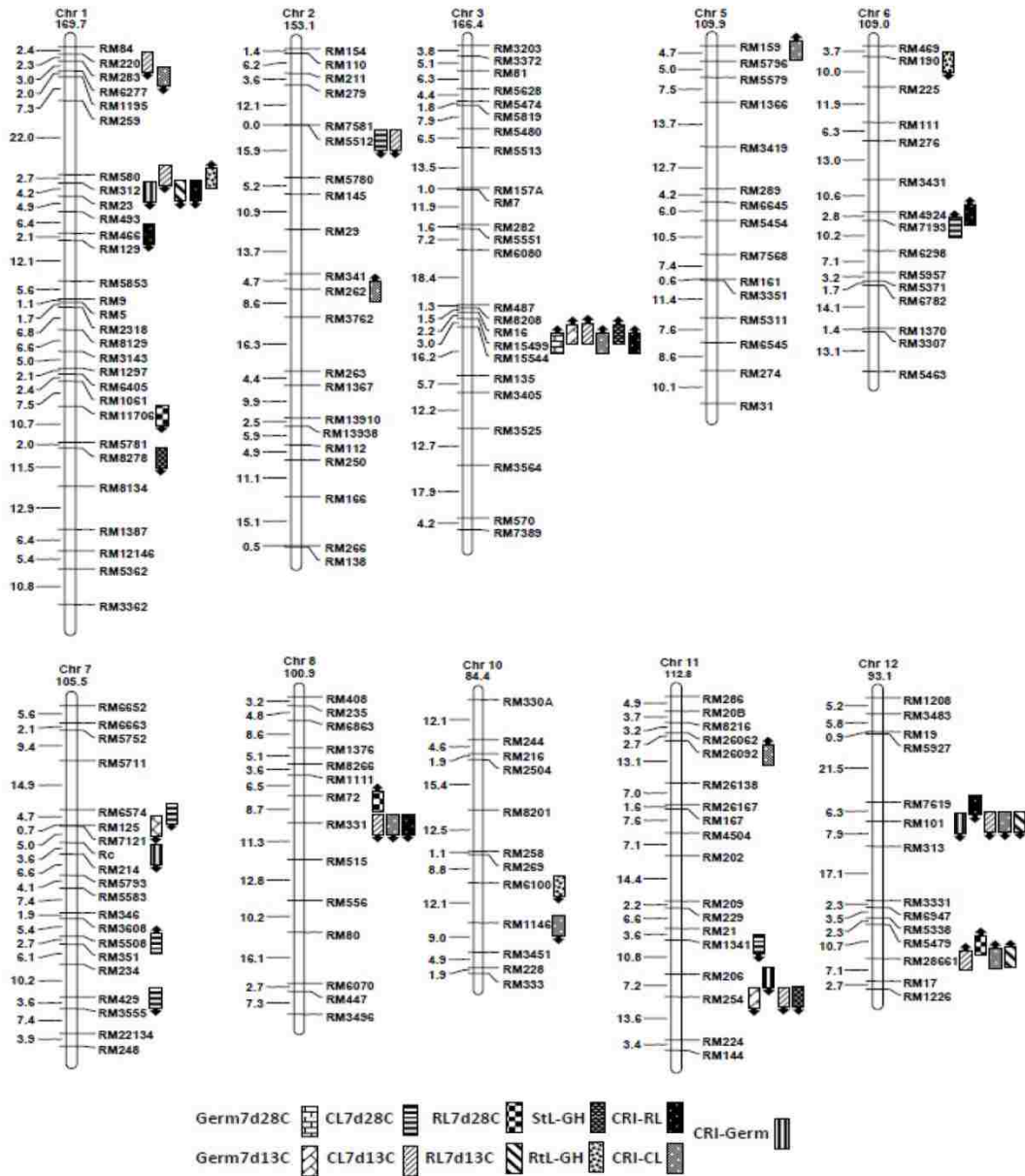
Figure 6. Chromosomal location of QTL for traits related to cold tolerance at germination stage and seedling vigor in the RIL population developed from the cross Bengal x PSRR-1 (Subudhi et al., 2012). QTL are shown on the left side of each chromosome. Bars with an arrow pointing upward indicates increasing effect on trait mean by Bengal allele and bars with down ward pointing arrow indicate PSRR allele increasing the trait mean. Germ7d28C, Arcsine transformed values of germination% after 7 days at 28°C; Germ7d13C, Arcsine transformed values of germination% after 7 days at 13°C; CRI-Germ, cold response index for germination; CL7d28C, Coleoptile length after 7 days at 28°C; CL7d13C, Coleoptile length after 7 days at 13°C; CRI-CL, cold response index for coleoptile length; RL7d28C, Radicle length after 7 days at 28°C; RL7d13C, Radicle length after 7 days at 13°C; CRI-RL, cold response index for radicle length; StL-GH, shoot length in greenhouse experiment; RtL-GH, root length in greenhouse experiment.

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Based on the radicle length and coleoptile length under normal condition of growth, PSRR-1 showed improved seedling vigor compared with Bengal. But the QTL mapping results for both traits under optimum environments revealed that both parents had genes or QTL, which can improve seedling vigor. For example, there were two QTL each for CL7d28C and RL7d28C with Bengal allele providing the alleles with positive effects. Other traits measured under cold stress followed a similar trend with both parents contributing alleles to improve germination ability and early seedling vigor under low temperature stress.

Table 3. Chromosome location, additive effects, direction of phenotypic effect, and estimate phenotypic variance of the putative QTL associated with early seedling stage cold tolerance and seedling vigor detected in the RIL population of the cross Bengal x PSRR-1 using composite interval mapping procedure

Trait [a]	QTL	Nearest Marker	Position[b]	LOD Value	Additive effect[c]	R^{2d}	DPE[e]
Germ7d28C	qGerm728-3	RM15499	96.4	4.1	3.465	7.6	B
Germ7d13C	qGerm713-3	RM16	92.2	3.683	4.673	5.1	B
	qGerm713-7	RM125	36.7	3.156	-4.746	4.9	P
	qGerm713-11	RM254	96.7	6.902	-6.860	9.5	P
CRI-Germ	qCRI-Germ-1	RM312	41.7	3.901	-6.417	5.4	P
	qCRI-Germ-7	Rc	44.4	2.526	-4.981	3.7	P
	qCRI-Germ-11	RM206	95.5	6.529	-8.283	9.6	P
	qCRI-Germ-12	RM101	39.7	2.631	-4.746	3.9	P
CL7d28C	qCL728-2	RM5512	38.3	2.640	-1.881	4.0	P
	qCL728-6	RM7193	58.3	2.580	1.795	3.7	B
	qCL728-7-1	RM6574	36.0	4.317	-2.581	6.8	P
	qCL728-7-2	RM5508	71.4	3.992	2.550	5.7	B

Table 3. continued

Trait[a]	QTL	Nearest Marker	Position[b]	LOD Value	Additive effect[c]	R^{2d}	DPE[e]
	qCL728-7-3	RM429	90.4	2.504	-1.941	3.5	P
	qCL728-11	RM1341	83.7	2.806	-2.066	4.8	P
CL7d13C	*qCL713-1-1*	RM220	4.4	3.538	-0.434	5.0	P
	qCL713-1-2	RM580	41.0	3.039	-0.337	4.1	P
	qCL713-2	RM5512	34.3	2.781	-0.347	5.1	P
	qCL713-3	RM16	93.2	6.751	0.473	9.7	B
	qCL713-8	RM331	43.5	5.600	-0.611	11.2	P
	qCL713-11	RM254	95.7	3.775	-0.344	5.0	P
	qCL713-12-1	RM101	39.7	5.979	-0.427	8.3	P
	qCL713-12-2	RM28661	88.5	6.101	0.478	9.4	B
CRI-CL	*qCRI-CL-1*	RM283	4.7	6.047	-1.106	8.3	P
	qCRI-CL-2	RM262	76.7	3.477	0.919	5.6	B
	qCRI-CL-3	RM15499	94.4	3.228	0.755	4.2	B
	qCRI-CL-5	RM159	0.0	3.176	0.747	4.1	B
	qCRI-CL-8	RM331	40.5	2.692	-0.776	3.6	P
	qCRI-CL-10	RM1146	69.5	4.016	-0.886	5.6	P
	qCRI-CL-11	RM26092	26.5	4.012	0.917	6.3	B
	qCRI-CL-12-1	RM101	39.7	4.808	-0.919	6.5	P
	qCRI-CL-12-2	RM28661	86.5	3.270	0.875	5.0	B
RL7d28C	*qRL728-1*	RM11706	117.2	5.726	-4.073	9.6	P
	qRL728-8	RM72	31.8	6.060	4.671	8.9	B
	qRL728-12	RM5479	80.8	3.137	3.017	5.0	B

Table 3. continued

Trait [a]	QTL	Nearest Marker	Position[b]	LOD Value	Additive effect[c]	R^{2d}	DPE[e]
RL7d13C	*qRL713-1*	RM312	41.7	3.003	-0.434	5.1	P
	qRL713-12-1	RM101	39.7	2.836	-0.427	4.9	P
	qRL713-12-2	RM28661	87.5	3.873	0.543	7.2	B
CRI-RL	*qCRI-RL-1-1*	RM312	41.7	5.124	-1.497	6.8	P
	qCRI-RL-1-2	RM466	56.8	3.764	-1.395	5.4	P
	qCRI-RL-3	RM15499	94.4	4.073	1.329	5.4	B
	qCRI-RL-6	RM4924	56.5	2.781	1.201	3.8	B
	qCRI-RL-8	RM331	41.5	4.724	-1.591	7.2	P
	qCRI-RL-12	RM7619	39.4	4.697	-1.419	6.5	P
StL-GH	*qStL-GH-1*	RM8278	130.9	6.834	-2.098	12.7	P
	qStL-GH-3	RM16	93.2	5.233	1.655	7.9	B
	qSL-GH-11	RM254	98.7	6.044	-1.951	11.1	P
RtL-GH	*qRtL-GH-1*	RM580	41.0	3.421	0.491	5.5	B
	qRtL-GH-6	RM190	3.7	2.878	-0.403	4.5	P
	qRtL-GH-10	RM6100	57.4	4.584	-0.529	7.9	P

[a] Germ7d28C, Arcsine transformed values of germination% after 7 days at 28°C; Germ7d13C, Arcsine transformed values of germination% after 7 days at 13°C; CRI-Germ, cold response index for germination; CL7d28C, Coleoptile length after 7 days at 28°C; CL7d13C, Coleoptile length after 7 days at 13°C; CRI-CL, cold response index for coleoptile length; RL7d28C, Radicle length after 7 days at 28°C; RL7d13C, Radicle length after 7 days at 13°C; CRI-RL, cold response index for radicle length; StL-GH, shoot length in greenhouse experiment; RtL-GH, root length in greenhouse experiment.
[b] QTL peak position on the linkage map.
[c] Additive effects of Bengal allele.
[d] Phenotypic variation (%) explained by each QTL.
[e] DPE, direction of phenotypic effect. B and P denote Bengal and PSRR-1 alleles increasing the phenotypic values, respectively.

2.4 Discussion

Low temperature in temperate and high altitude areas of the tropical regions is a major hindrance to rice cultivation. It has a detrimental effect on germination, seedling growth, and reproductive success by interfering with the metabolism (Zhang et al., 2014). Since tolerance to low temperature at a particular stage of crop growth is not correlated to cold tolerance at other developmental stages (Kaw & Khush, 1985; Zhou et al., 2012), QTL studies had been conducted to investigate cold tolerance at various growth and developmental stages such as germination stage (Miura et al., 2001; Fujino et al., 2004), seedling stage (Kim et al., 2014; Zhang et al., 2014; Liu et al., 2015), vegetative stage (Andaya & Mackill, 2002), and booting stage (Andaya & Mackill, 2003; Oh et al., 2004). In this study, we focused on germination ability and early seedling growth under cold stress, which is an important factor for uniform stand establishment in temperate rice growing areas of southern USA, where cold temperature prevents germination in dry seeded rice.

There was no difference in germination rate under optimum temperature. Therefore, it was obvious that we detected only one QTL from the cultivated rice suggesting little genetic difference between both parents with respect to germination ability at optimum temperature. However, there was a strong contrast between both parents for germination ability under cold stress, which was supported by detection of four QTL on chromosomes 1, 7, 11, and 12 controlling this trait and PSRR-1 alleles in all cases were superior to Bengal (Table 3). The reduction in coleoptile length and radicle length at cold temperature was higher compared to observations in Japanese rice germplasm (Bosetti et al., 2012).

Since PSRR-1 was superior to Bengal with respect to the coleoptile length and radicle length under optimal temperature regime (Table 1), it provided an opportunity to identify the

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genomic regions responsible for such attributes related to seedling vigor. There was no colocalisation of the QTL for coleoptile length and radicle length indicating different genes responsible for these traits. The QTL alleles from both parents contributed to the improvement of seedling vigor under optimum temperature.

Comparison of QTL positions on the rice genome with those detected in prior studies provided information about the QTL consistency despite the differences in marker types, the genetic materials, and screening methodology. Miura et al. (2001) reported five QTL on chromosomes 2, 4, 5, and 11 controlling germination ability under cold stress. Three of these QTL on chromosomes 2, 5, and 11 were located in similar positions in our study. Fujino et al. (2004) identified 3 QTL on chromosomes 3 and 4 with one major QTL *qLTG-3-1* explaining 35% of phenotypic variation, which was later cloned (Fujino et al., 2008). However, we did not detect any QTL on corresponding locations of both chromosomes. Baruah et al. (2009) reported five QTL for cold tolerance at the plumule and seedling stage in a cross between a *japonica* rice A58 and a wild rice accession W107 (*Oryza rufipogon*). The QTL on chromosome 11, which was consistent at plumule and seedling stages, also overlapped with the same genomic region harboring several QTL for cold tolerance attributes in our study (Fig. 6). Few other studies reported 2-5 QTL or genomic regions controlling these attributes (Chen et al., 2006; Zhang et al., 2005b; Ranawake et al., 2014). There was no overlapping of QTL detected in our study with those identified by Chen et al. (2006). Three QTL on chromosomes 6 and 7 for germination ability under cold stress and two QTL on chromosomes 6 and 11 for cold tolerance at seedling stage (Ranawake et al., 2014) coincided with our study. Several studies reported QTL for cold tolerance traits such as seedling vigor (Zhang et al., 2005a; Baruah et al., 2009), tolerance to seedling stage necrosis (Andaya & Mackill, 2003), and seedling stage cold tolerance (Kim et al.,

2014) in the similar region on chromosome 11. Considering all these reports, it is clear that this chromosome 11 location might be a hot spot for genes responsible for cold tolerance at germination and seedling stages. A candidate gene, ORF LOC_Os11g37720 (*Duf6* gene), co-segregating with seedling cold tolerance was reported (Kim et al., 2014). Further exploration of this region using the introgression line PSRR-1 (Subudhi et al., 2015) should lead to cloning of the genes responsible for various component traits of cold tolerance.

Mapping of seedling vigor, which was assessed in both laboratory and greenhouse condition revealed that genes controlling seedling vigor under cold stress were different from those under normal temperature (Fig. 6). When QTL results for all traits were considered in totality, we observed that there were six clusters of QTL for multiple seedling vigor and germination traits under cold stress on chromosomes 1, 3, 8, 11, and 12. For the two clusters on chromosomes 3 and 12, positive alleles for all cold tolerance traits were from 'Bengal', whereas weedy rice alleles improved cold tolerance in the remaining 4 clusters. Strong correlations observed among the traits (Table 2) were consistent with QTL mapping results because the majority of QTL for both germination ability and seedling vigor traits were colocalized in few chromosomal regions with the same parental allele either decreasing or increasing the trait mean (Fig. 6; Table 3). It is interesting to note that most of the congruent QTL were not localized in the QTL clusters detected in this study with the exception of that on chromosome 11. It appears that these QTL are novel compared with the earlier reports (Miura et al., 2001; Fujino et al., 2004; Zhang et al., 2005b; Chen et al., 2006; Ranawake et al., 2014). Most of these studies used *indica* x *japonica* cross with the exception of Fujino et al. (2004), who used the population developed from the cross between two *japonica* varieties. Unlike the above studies, the mapping population used in our study involved a *japonica* variety and a weedy rice accession. Therefore,

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the discrepancy in QTL detection could be due to the differences in the genetic background of the plant materials. Zhang et al. (2005a) reported five genomic regions where QTL for multiple traits were evaluated to assess seedling vigor. Each cluster was designated as a putative QTL, which could be either cluster of linked loci or single locus with pleiotropic effects. Another finding was that two StL-GH QTL and one RtL-GH QTL were placed in these clusters along with other seedling and germination attributes, which suggest that these traits could be used as reliable indicators to evaluate seedling vigor under cold stress (Zhang et al., 2005a).

The weedy rice accession PSRR-1 was collected from the rice field in Louisiana, USA, where cold stress is common during the rice cropping season. High level of seed germination ability under cold stress in this weedy rice accession might be due to strong selection pressure for cold tolerance and early seedling vigor (Mackill & Lei, 1997; Baruah et al., 2009). Weedy rice populations from different geographic location of China have been reported to evolve rapidly with respect to their germination response due to genetic differentiation (Chen et al., 2004; Xia et al., 2011). Despite many undesirable attributes, weedy rice could be an important resource for genetic improvement of crop plants like other wild relatives (Lu & Ellstrand, 2014). We showed that the four QTL clusters on chromosomes 1, 8, 11, and 12, where weedy rice alleles were beneficial, would be useful for marker-assisted selection to introduce a high level of cold tolerance and seedling vigor to rice cultivars grown in the USA. Since we observed transgressive variation for both improved germination and vigor under cold stress in the mapping population, it is likely that these QTL alleles from weedy rice in combination with the favorable alleles of the cultivated rice would result in rice varieties with improved cold tolerance. Consequently, cloning of these QTL using the introgression lines of this weedy rice accession developed in a

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cultivated background (Subudhi et al., 2015) should aid in the precise transfer of the cold

tolerance attributes from the weed rice.

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Chapter 3. Genetics of Cold Tolerance at Seedling Stage in a RIL Population Involving Weedy Rice

3.1 Introduction

Low-temperature stress is an important factor limiting growth, development, and productivity of rice (*Oryza sativa* L.) in high altitudes in tropical, sub-tropical and temperate regions. Over 15 Mha of rice throughout the world is affected by cold damage at one or more growth stages (Zhang et al., 2014b). Rice seedlings are generally susceptible to temperatures below 15-17°C (Yoshida et al., 1996). Cold stress is known to cause negative effects on rice, particularly when the crop is sown by direct seeding method.

Depending on the geographic locations and climatic factors, rice plants may experience cold stress at various developmental stages: germination stage, seedling stage, and reproductive stage. Cold stress during seedling stage reduces seedling growth leading to delayed flowering and poor grain filling (Andaya & Tai, 2006). Cold stress induced damage is manifested as poor seedling establishment, slow growth, chlorotic leaves, reduced tillering and eventually, seedling death (Kaneda & Beachell, 1974). Several criteria have been adopted as a criterion to evaluate the level of cold tolerance at the seedling stage of rice. These are seedling survival rate (Lou et al., 2007; Qian et al., 2000; Zhang et al., 2005a), necrosis or wilting (Andaya & Mackill, 2003; Andaya & Tai, 2006), leaf yellowing and stunting (Andaya & Tai, 2006), arbitrary rating scale (Misawa et al., 2000), shoot length, root length, and seedling dry mass (Zhang et al. 2005a). Screening for cold tolerance under natural conditions as well as in controlled environments has been suggested to minimize the uncertainty of low temperature patterns in field conditions (Jiang et al., 2011). For genetic investigation of cold tolerance in rice, screening should be performed at

different developmental stages due to absence of correlation of cold tolerance between different stages of crop growth (Kaw & Khush, 1985).

Development of rice varieties with increased tolerance to cold stresses is an important breeding objective in regions where low temperatures prevail during growing season. Cold tolerance is a genetically complex trait due to involvement of multiple genes and their interaction with environmental factors (Ranawake et al., 2008). Identification of QTLs is an effective approach to understand the genetic basis controlling this trait. Most of the mapping populations in earlier studies were derived from *indica/japonica* crosses and *japonica* cultivars were more cold tolerant than *indica* cultivars (Andaya & Mackill, 2003).

Genetics of seedling cold tolerance has been investigated and several QTLs associated with this trait have been identified (Zhang et al., 2014a). Using an RIL population, Andaya & Mackill (2003) identified 11 QTLs that were distributed on eight chromosomes and fine mapping of the major effect QTL led to identification of two candidate genes *OsGSTZ1* and *OSGSTZ2* (Andaya & Mackill, 2006). Another QTL, *qCTS4*, associated with tolerance to yellowing and stunting of rice seedlings, was mapped to chromosome 4 using the same RIL mapping population (Andaya & Tai, 2007). Using seedling survival percentage after cold treatment for 10 and 13 days at 10°C as a criterion, three QTLs were identified of which *qSCT-11* was a major QTL with 30% of the phenotypic variation (Zhang et al., 2005b). Another major QTL *qCTS-2* on chromosome 2 with 27% of phenotypic variation was identified in a doubled haploid population (Lou et al., 2007). In an F_2 population developed from the cross between cold tolerant *Oryza rufipogon* accession W1943 and a sensitive *indica* cultivar GLA4, Koseki et al. (2010) identified a major seedling stage QTL *qCtss11* and the candidate gene Os11g0615900 underlying it. In two $F_{2:3}$ mapping populations from crosses involving a cold tolerant land race Xiang743 and two cold

susceptible varieties (Katy and Dular), Liu et al., (2015) used seedling survival % as a criterion

of cold tolerance and identified 5 and 7 QTLs in Xiang/Dular and Xiang743/Katy population,

respectively. All congruent QTLs were mapped in the same chromosomal location in both

populations and a major QTL on chromosome 8 contributed 14% and 39% of the phenotypic

variation in Xiang 743/Katy and Xiang 743/Dular populations, respectively.

In the U.S., Louisiana is the third leading rice producing state with 28.4 million cwt of

rice produced in 2016 (USDA National Agricultural Statistics Service, 2017). The Rice Research

Station of the Louisiana State University Agricultural Center has made tremendous contribution

to the rice industry with development of several high yielding rice varieties. Since low

temperature stress during the planting season is a major constraint for early planting in

Louisiana, development of cold tolerant varieties is an effective strategy to reduce the negative

impact of cold stress on rice plant growth, development, and productivity. In this study, a high

yielding rice cultivar "Bengal" was crossed to a Louisiana weedy rice accession PSRR-1 to

develop a recombinant inbred line population for mapping QTL for seedling stage cold tolerance.

The objective of this study was to investigate the genetics of cold tolerance at the seedling stage

of rice.

3.2 Materials and Methods

3.2.1 Plant Materials

This study was carried out at the seedling stage of rice. The plant materials consist of a

population of 198 RILs in the $F_{7:8}$ generation developed from the cross between a medium grain

high yielding rice cultivar 'Bengal' (Linscombe et al., 1993) and a weedy rice accession PSRR-1

(Subudhi et al., 2012). PSRR-1 was collected from the Rice Research Station at Crowley, LA

and was purified by single plant selection for two generations before making crosses to develop

the mapping population. It has light green leaves, vigorous growth, long auricles and ligules, straw-hulled medium grains, lax open panicles, and pubescent leaves. This weedy rice accession is extremely susceptible to shattering and has a higher intensity of both hull and pericarp dormancy compared to Bengal.

3.2.2 Evaluation of Cold Tolerance at Seedling Stage

For each line and parents, seeds were treated at 50 °C for 5 days to eliminate residual dormancy. Ten seeds of each line and parents were sown in soil-filled gardening pots (6cm x 4cm x 5.7cm). A mixture of river silt soil with Miracle-Gro® potting mix in a 2:1 ratio was used to prepare the soil. The pots were submerged with a water depth of 2cm during the whole project duration. The experiment was conducted in a randomized block design with two replications. Seedlings were allowed to grow until the third-leaf stage (approx. 21 days) in the greenhouse (14-hour photoperiod). Seedlings were then exposed to 18/8°C day/night temperature for 18 days with 12-hour photoperiod in a growth chamber. When significant visual damage was observed between parents, the effect of low temperature was scored on 5 randomly selected seedlings using the cold injury score scale (Table 4).

Table 4. Cold injury score scale

Score	Observation	Level of tolerance
1	No visual symptoms.	Highly tolerant
3	Leaf tips or few leaves whitish and rolled.	Tolerant
5	Most leaves rolled; only few are elongated.	Moderately tolerant
7	Most leaves dry; plants nearly dying.	Susceptible
9	All plants are wilting or dead.	Highly susceptible

After cold treatment, seedlings were transferred to the greenhouse for a 7-day recovery period. After the recovery period, they were screened again for cold injury score, shoot length and root length to evaluate differences in performance.

3.2.3 Statistical Analyses

Analysis of variance (ANOVA) and mean comparison were done using the GLIMMIX procedure. Lines were entered as the fixed effect and the replications were treated as random effect. Pearson correlation coefficients among traits were computed based on RIL means using the CORR procedure and heritability of each trait was estimated following Holland et al. (2003). All analyses were carried out using SAS (SAS Institute, 2011). Histograms were made in Microsoft Excel 2010.

3.2.4 QTL Analysis

A linkage map developed in this RIL population (Subudhi et al., 2012) was used for QTL mapping. The linkage map consisted of 212 simple sequence repeat (SSR) markers and a morphological marker *Rc* (the pericarp color) with a total map distance of 1410 cM and average marker interval of 6.6 cM. Both single marker analysis and composite interval mapping (CIM) were performed using QTL Cartographer version 2.5 (Wang et al., 2011). In the CIM procedure, a forward-backward regression procedure with 20 cofactors was followed with walk in speed of 1.0 cM for detection of QTL. Logarithm of odds (LOD) score of 2.5 was used as the threshold for declaring significance of the QTL. Since QTL identified using data taken after 14 days of exposure at 13ºC were same as those from 7 days at 13ºC, those data were not provided. The QTL were named following McCouch et al. (1997). For example, the QTL located on chromosome 2 for cold injury score after cold stress was named as *qCIS (CS)-2*.

3.3 Results

3.3.1 Variation for Seedling Stage Cold Tolerance

The parents and RIL population were evaluated under cold stress at seedling stage for cold injury score after cold stress (CIS-ACS), cold injury score after 7 days of recovery period (CIS-ARP), shoot length after 7 days of recovery period (StL-ARP), and root length after 7 days of recovery period (RtL-ARP). Bengal and PSRR-1 showed significant difference for CIS-ACS but not for CIS-ARP, StL-ARP, and RtL-ARP (Table 5). There was wide range of variation in CIS (CS) (Fig. 7). Bengal showed lower CIS-ACS and CIS-ARP indicating its superiority over PSRR-1. Mean values of the RIL population were similar to that of Bengal for CIS-ACS and CIS-ARP whereas StL-ACS, StL-ARP and RtL-ARP values of RIL population were lower than either parent.

Among the RILs, there were significant genotypic differences for traits, CIS-ACS and StL-ARP (Table 5). Heritability was high for StL-ARP, moderate for CIS-ACS, and very low for CIS-ARP and RtL-ARP. Frequency distribution was normal for all traits except for CIS-ARP and several lines were phenotypically superior than either parent for these traits (Fig. 8 and 9).

Table 5. Descriptive statistics for cold tolerance-related traits during seedling stage in Bengal x PSRR-1 RIL mapping population of rice

Trait	Bengal mean	PSRR-1 mean[a]	BR-RIL mean	Std. Dev.	BR-RIL range	Pr>F[b]	Heritability
CIS-ACS	5.00	7.30[**]	5.10	1.09	2.60-8.40	0.0291	0.34
CIS-ARP	7.80	8.90[ns]	7.99	1.12	5.00-9.00	0.7186	0.08
StL-ARP (cm)	24.60	23.80[ns]	21.89	4.77	7.00-38.80	<0.0001	0.82
RtL-ARP (cm)	12.40	12.40[ns]	11.32	1.45	6.00-15.50	0.2903	0.07

CIS-ACS, cold injury score after cold stress at 18/8 °C day/night temperature; CIS-ARP, cold injury score after 7 days of recovery period in greenhouse; StL-ARP (cm), shoot length after 7 days of recovery period in greenhouse; RtL-ARP (cm), root length after 7 days of recovery period in greenhouse.
[a] Difference in trait means between Bengal and PSRR-1; [b] Significant difference among the RILs of the Bengal x PSRR-1 mapping population based on F test from the ANOVA; [ns] not significant; [**] Significant at P<0.01;
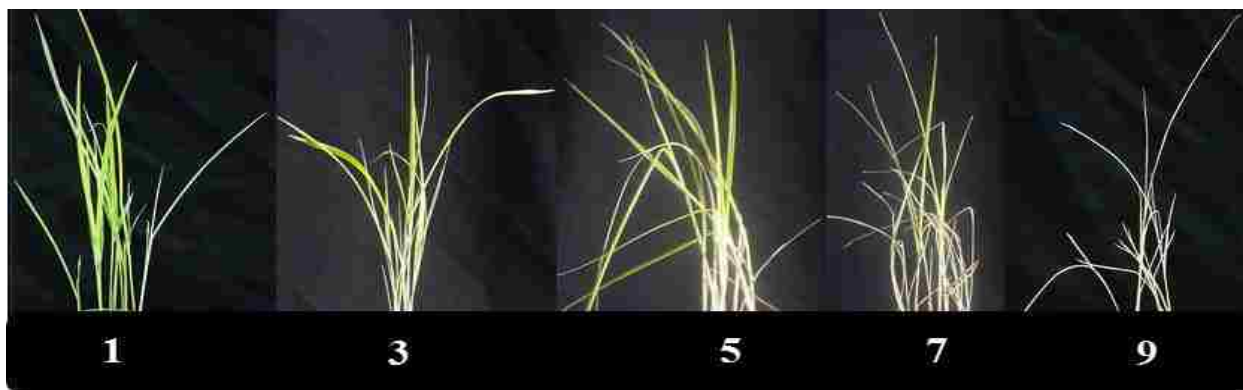
Figure 7. Performance of RILs for cold injury scores. Cold injury was scored after 18 days of cold exposure at 18/8 °C.
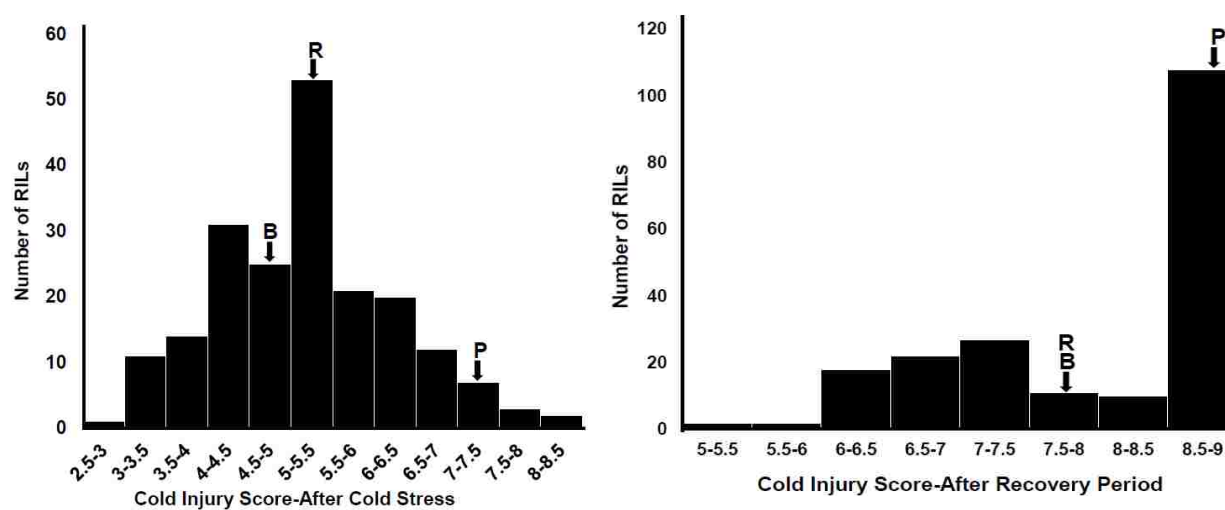
Figure 8. Frequency distribution of cold injury score after cold stress and after 7 days of recovery period in greenhouse in RIL population of the cross Bengal x PSRR-1. The seedlings were transferred to greenhouse after the cold tolerance evaluation in the laboratory. Mean values for parents and RIL population are marked by arrows. B, P, and R represent for Bengal, PSRR-1, and RIL population. The cold injury score (CIS) is indicated in a scale of 1-9, where 1=highly tolerant, 3=tolerant, 5=moderately tolerant, 7=susceptible and 9=highly susceptible.

Figure 9. Frequency distribution of shoot length and root length after 7 days of recovery period in greenhouse in RIL population of the cross Bengal x PSRR-1. The seedlings were transferred to greenhouse after the cold tolerance evaluation in the laboratory. Mean values for parents and RIL population are marked by arrows. B, P, and R represent for Bengal, PSRR-1, and RIL population. The cold injury score (CIS) is indicated in a scale of 1-9, where 1=highly tolerant, 3=tolerant, 5=moderately tolerant, 7=susceptible and 9=highly susceptible.
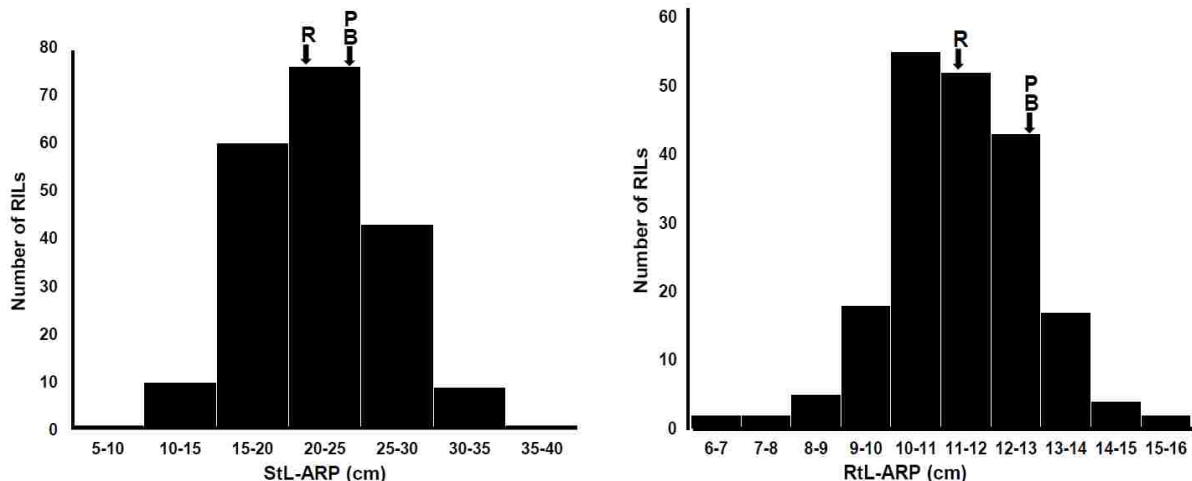
3.3.2 Correlation among traits

As presented in Table 6, both positive and negative phenotypic correlations among the seedling stage cold tolerance traits were noticed. There were negative and significant correlations between RtL-ARP and rest of the traits. On the other hand, CIS-ARP was significant and positively correlated to CIS-ACS and StL-ARP.

Table 6. Pearson correlation coefficients of seedling stage cold tolerance traits in the RIL mapping population of rice cross Bengal/PSRR-1

	CIS-ACS	CIS-ARP	StL-ARP	RtL-ARP
CIS-ACS	1.000			
CIS-ARP	0.418***	1.000		
StL-ARP	-0.025	0.178***	1.000	
RtL-ARP	-0.163**	-0.238***	-0.112*	1.000

Significance of correlation coefficients were indicated by *(p values>0.05), ** (p values>0.01), and ***(p values>0.001)

3.3.3 Quantitative trait loci for cold tolerance at seedling stage under cold stress

Scanning of the whole genome detected 23 QTL for four traits distributed along all twelve chromosomes under cold stress environments (Table 7 and Fig. 10). The number of QTL varied between four and eight per trait and each QTL contributed 2 to 42% of the total phenotypic variation. Bengal alleles contributed toward increased trait means in case of 10 QTLs and PSRR-1 alleles were responsible in rest 13 QTLs.

Five chromosomal regions with significant additive effect were detected for CIS-ACS on chromosomes 2, 6, 8, and 11. All detected QTL had small effects ranging from 4 to 8% of the phenotypic variation. In case of three QTLs [*qCIS(CS)-2, qCIS(CS)-6* and *qCIS(CS)-8.2*] increasing trait means were due to Bengal alleles. Since lower CIS values were desirable, PSRR-1 alleles were desirable in these three QTLs whereas Bengal alleles were responsible for enhancing cold tolerance in remaining two QTLs.

For CIS-ARP, a total of eight chromosome regions were detected on chromosome 3, 4, 10, 11, and 12. All additive QTLs were minor-effect QTL with less than 10% of the total phenotypic variation. Bengal alleles were responsible for enhancing cold tolerance in four of the eight QTLs. For CIS-ARP and CIS-ACS, only one QTL on chromosome 11 was common.

Composite interval mapping revealed 6 QTLs on chromosomes 1, 7, and 9 for shoot length after recovery period. *qStL(RP)-1.3* was identified as a major QTL with LOD value of 28.1 and 42% of the phenotypic variation. This QTL may be colocalizing with the semidwarf (*Sd1*) locus. The additive effect of this QTL had increasing effect due to PSRR-1 allele. For only one QTL *qStL(RP)-1.4*, Bengal allele was responsible for increased seedling height. RtL-ARP was controlled by four QTL located on chromosome 5, 7, and 8. All additive QTL were minor-

Table 7. Quantitative trait loci associated with seedling stage cold tolerance in the RIL population of the cross Bengal x PSRR-1 using composite interval mapping procedure.

Trait[a]	QTLs	Peak marker	Position[b]	LOD value	Additive effect[c]	R^{2d}	DPE[e]
CIS(CS)	qCIS(CS)-2	RM13910	112.9	5.45	0.296	7.1	B
	qCIS(CS)-6	RM3431	44.9	5.67	0.393	7.8	B
	qCIS(CS)-8.1	RM515	51.8	4.12	-0.271	5.4	P
	qCIS(CS)-8.2	RM3496	99.6	2.50	0.217	3.5	B
	qCIS(CS)-11	RM206	86.7	3.39	-0.257	5.0	P
CIS(RP)	qCIS(RP)-3.1	RM487	89.4	3.10	0.272	4.4	B
	qCIS(RP)-3.2	RM3564	145.2	3.72	0.293	6.1	B
	qCIS(RP)-4	RM3742	40.5	2.83	-0.238	4.1	P
	qCIS(RP)-10.1	RM6100	55.6	4.11	-0.385	6.8	P
	qCIS(RP)-10.2	RM1146	68.5	5.49	0.428	8.3	B
	qCIS(RP)-11	RM206	86.7	2.62	-0.233	4.1	P
	qCIS(RP)-12.1	RM101	41.7	3.69	-0.340	6.7	P
	qCIS(RP)-12.2	RM5479	72.5	5.28	0.408	8.2	B
StL(RP)	qStL(RP)-1.1	RM220	2.4	2.70	-0.983	2.5	P
	qStL(RP)-1.2	RM466	57.2	3.39	-1.234	3.2	P
	qStL(RP)-1.3	RM8278	128.9	28.1	-3.679	42.1	P
	qStL(RP)-1.4	RM1387	147.3	3.89	1.208	3.6	B
	qStL(RP)-7	Rc	42.4	2.62	-0.854	2.4	P
	qStL(RP)-9	RM107	72.2	2.84	0.810	2.7	P
RtL(RP)	qRtL(RP)-5	RM274	100.9	3.19	-0.377	6.1	P
	qRtL(RP)-7	RM6652	1.0	3.53	0.458	6.8	B
	qRtL(RP)-8.1	RM6863	10.0	3.59	-0.436	7.5	P
	qRtL(RP)-8.2	RM72	35.8	2.73	0.401	6.6	B

[a] CIS(CS), cold injury score after cold stress; CIS(RP), cold injury score after recovery period; StL(RP), shoot length after recovery period; RtL(RP), Root length after cold stress
[b] QTL peak position on the linkage map
[c] Additive effects of Bengal allele
[d] Phenotypic variation (%) explained by each QTL
[e] DPE, direction of phenotypic effect. B and P denote Bengal and PSRR-1 alleles increasing the phenotypic values, respectively
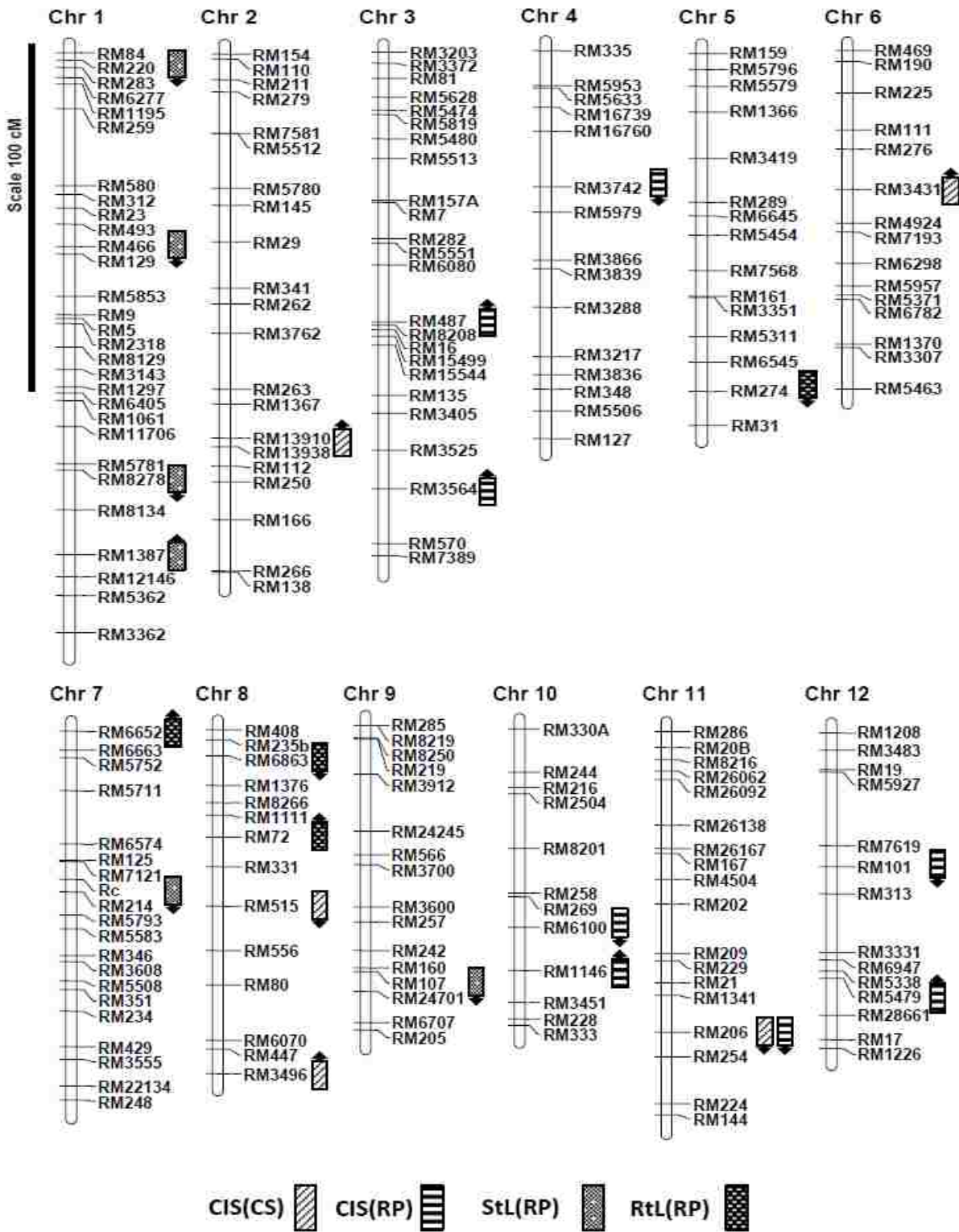
Figure 10. Chromosomal location of quantitative trait foci (QTL) for seedling stage cold tolerance in the recombinant inbred line (RIL) population from the cross between Bengal and PSRR-1 (Subudhi et al. 2012).

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effect QTL. Bengal alleles were responsible for increased phenotypic effect in case of two QTL and PSRR-1 in the remaining two.

3.4 Discussion

Breeding of rice varieties with tolerance to low temperature at early growth stages is an important breeding objective. Cold tolerant rice varieties ensure uniform seedling establishment (Yamamoto, 1990). Due to the complexity of symptoms associated with low-temperature stress, screening for cold tolerance at the seedling stage is difficult to accomplish in a single test or considering it as a single trait. Previous studies (Andaya & Mackill, 2003; Jiang et al., 2008; Lou et al., 2007; Kim et al., 2014) used to score cold tolerance based on the degree of chlorosis, stunting, and seedling mortality. In this study, cold tolerance at the seedling stage was evaluated based on visual symptoms, shoot length, and root length after exposure to cold stress and recovery period.

Based on cold injury score, this study demonstrated that Bengal is more tolerant to cold stress at seedling stage compared to the weedy rice PSRR-1 (Fig. 7, Table 5), whereas PSRR-1 performed better at germination stage (discussed in chapter 2). This observation suggests that there are different sets of genes responsible for cold tolerance at both developmental stages. However, comparison of the QTL locations for cold tolerance QTL of both stages revealed some degree of congruency. For example, QTL for trait CL7d13C at germination stage overlapped with QTLs for CIS (CS) and CIS (RP) on chromosomes 3, 8, 11, and 12. There was clustering of germination stage cold tolerance QTLs in these regions. As we have discussed earlier, there are desirable alleles in both parents, emphasis should be given to introgress the desirable weedy rice alleles for improving cold tolerance.

There are several QTL mapping studies on seedling stage tolerance in rice (Andaya &

Mackill, 2003; Lou et al., 2007; Baruah et al., 2009; Kim et al., 2014; Yang et al., 2016). As

expected, there was large discrepancy in QTL positions and their effects which could be due to

use of different type of plant materials and different screening methodology. Detection of only

minor-effect QTLs on controlling seedling stage cold tolerance trait (Table 7, Fig. 10) indicated

complex inheritance with large influence of environmental factors. Since lower CIS values were

desirable, PSRR-1 alleles were responsible for high cold injury scores in case of three QTLs for

CIS (CS) whereas Bengal alleles were responsible for increased cold tolerance in remaining two

chromosomal regions. Composite interval mapping detected one common QTL on chromosome

11 for cold injury score after cold stress and recovery period. This QTL on chromosome 11

overlapped with cold tolerance QTL reported previously using different genetic background and

screening methodology (Andaya & Mackill, 2003; Baruah et al., 2009; Kim et al., 2014). Since

this region seems to be hot spot for cold tolerance genes for both germination stage (chapter 2)

and seedling stage in this study as well as in above studies, further exploration could lead to

identification of candidate genes for enhancing seedling cold-tolerance in rice.

One major QTL for shoot length of rice seedlings was detected on the long arm of

chromosome 1 [*qStL(RP)-1.3*] and weedy rice allele was responsible for increased seedling

height. This QTL was located in the same region as *qSL1* detected by Fukuda and Terao (2015).

Although there are genes affecting seedling height in this region such as *D61* (Yamamuro et al.,

2000), *SPS* (Ishimaru et al., 2004), *D10* (Arite et al., 2007), *CIGR* (Kovi et al., 2011), *THIS1*

(Liu et al., 2013), *Psd1* (Li et al., 2014), and *Sd1* (Sasaki et al., 2002), the green revolution gene

Sd1 encoding for gibberellin 20 oxidase-2 [OsGA20ox2] may be the most likely candidate. It has

been well characterized for its role in semi-dwarf plant characteristics (Sasaki et al., 2002).

Nonfunctional allele of this gene is responsible for semi-dwarf plant type. One study carried out at both optimum condition (28°C) and cold stress condition (16°C) suggested that *qStL(RP)-1.3* region affects the shoot growth rate under low-temperature conditions as well as in optimum environmental conditions.

Regarding the root length, no significant differences were found between the parental germplasm. This might be caused by environmental and physical conditions since the rice seedlings were grown in small pots with limited space. Also, this trait was negatively and significantly correlated to the rest of the traits, indicating the negative effect of low temperature on the overall growth of the plants. Our QTL mapping detected four small effect QTLs on chromosome 5, 7, and 8 with desirable alleles from both parents. Although Zhang et al. (2005a) identified four QTLs on chromosomes 5, 6, 8 and 12 based on root length; none of these QTLs overlapped the location of QTLs found.

There was only one seedling stage cold tolerance QTL mapping study that used a mapping population involving a wild rice *Oryza rufipogon* (Koseki et al., 2010). Three QTLs were identified on chromosomes 3, 10, and 11 with the large effect QTL on chromosome 11. Since the marker type used in their study was different from ours, it was difficult to compare the exact location of QTLs. But the visual examination of map indicated that the CIS (RP) QTLs were detected on same chromosomes may be congruent to those detected by Koseki et al. (2010).

Improving seedling stage cold tolerance is challenging because majority of QTLs for seedling stage cold tolerance are of minor effects. Moreover, despite a poor performance under cold stress at seedling stage, PSRR-1 alleles of some QTLs were responsible for increasing tolerance to cold stress at this developmental stage. Therefore, pyramiding of these favorable alleles for both seedling and germination stage cold tolerance from weedy rice will be necessary

to develop rice varieties with cold tolerance at both stages as demonstrated by Yang et al. (2016)

using single segment substitution lines. The availability of introgression lines of PSRR-1 in

Bengal varietal background (Subudhi et al., 2015), pyramiding of these QTLs should be possible

by crossing the ILs with desirable QTLs for cold tolerance at both developmental stages.

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Chapter 4. Summary and Conclusions

Improvement in cold tolerance of the rice cultivars is an important breeding objective. Prevalence of low temperature during planting and early part of the growing season is a major constraint for rice production in rice growing areas of the United States. Delayed germination coupled with reduced seedling vigor hinders crop establishment and crop growth resulting in reduced rice productivity. Mapping of the chromosomal regions controlling cold tolerance will be helpful to apply marker-assisted selection for development of cold tolerant rice varieties.

This study was undertaken to demonstrate that genetic attributes of weedy rice can contribute to develop new rice varieties with enhanced low temperature tolerance. Cold tolerance studies have been conducted in rice at different stages of the plant using *indica* and *japonica* rice cultivars. However, few studies have considered weedy rice as a suitable genetic resource to improve cold tolerance in rice. Bearing this in mind, we evaluated cold tolerance in 198 RILs at the germination and seedling stage. The mapping population was developed from a *japonica* cultivar 'Bengal' and the weedy rice accession 'PSRR-1'. Phenotypic evaluation at the germination stage was done under optimum temperature (28°C) and low temperature (13°C).

Germination percentage, coleoptile length, and radicle length were used as criterion of evaluation. Traits were measured at 7 days under optimum conditions whereas measurements were taken at 7 and 14 days under cold stress conditions. Our results indicated that, at optimum temperature germination percentage was not altered in any of the parent. However, when cold stress was imposed, PSRR-1 had higher germination percentage compared to Bengal. Negative effect of low temperature was noticeable in coleoptile and radicle growth. It is important to mention that under optimum conditions, PSRR-1 also showed its superiority in terms of coleoptile length and radicle length. After evaluation under controlled conditions in the

laboratory, cold stressed seeds were transferred to greenhouse facilities to assess growth and development of the plants in soil. Development was measured based on shoot and root length. Shoot length of PSRR-1 was higher than Bengal. Our phenotypic evaluation suggested that weedy rice could be a significant genetic source for cold tolerance rice breeding.

For genetic analysis of cold tolerance, composite interval mapping was carried out to identify QTLs conferring cold tolerance at the germination stage. A total of 49 QTLs were detected along ten chromosomes. Each QLT contributed 3.5 to 12.7% of the total phenotypic variation. Out of the 49 QTLs detected, PSRR-1 alleles were responsible for increasing cold tolerance at germination stage in 31 of them.

For evaluation at the seedling stage of rice, seedlings were allowed to grow until the 3rd leaf stage in the greenhouse and then transferred to a growth chamber adjusted at 18/8°C day/night temperature. Measurements were taken until cold injury was evident between the parents. Cold tolerance was measured based on cold injury score after cold stress as well as recovery period, root length, and shoot length. Bengal performed better than PSRR-1 based on the cold injury score after cold stress exposure. However, no difference between both parents was observed after the recovery period. A QTL on chromosome 11 [*qCIS(CS)-11*] was identified for cold injury after stress exposure as well as recovery period. Interestingly, QTLs responsible for conferring cold tolerance at germination stage were detected in the same region of this chromosome suggesting presence of genes responsible for cold tolerance at both developmental stages of the plant. Our study thus indicated that cold tolerance is developmentally regulated as well as growth stage-specific, and that cold tolerance at one growth stage is not necessarily correlated with cold tolerance at other stages.

Since the weedy rice has many undesirable characteristics, molecular markers tightly linked to desirable traits such as cold tolerance will be required to avoid linkage drag. Therefore, fine mapping of the QTLs should be undertaken using the introgression lines of the weedy rice accession PSRR-1 to determine the exact position of the putative QTLs and develop markers flanking the region for use in marker-assisted backcrossing. Alternatively, effort should be made to clone these QTLs for developing gene-based markers for precise introgression of cold tolerance QTLs to high yielding rice cultivars in the breeding program.

In conclusion, US rice variety Bengal lack high degree of cold tolerance at germination stage. Our study demonstrated that weedy rice accession PSRR-1 is superior to the US cultivar Bengal in tolerance to cold stress at germination stage. Despite its poor performance under cold stress at seedling stage, PSRR-1 alleles in some QTLs can be useful. Since the QTLs detected in this study are largely minor effect QTLs, pyramiding of the desirable QTLs from PSRR-1 to Bengal and other US cultivars may improve cold tolerance at both the developmental stages.

Appendix. Permit to reprint content of Borjas, A.H., De Leon, T.B., and Subudhi, P.K. (2016). Genetic analysis of germinating ability and seedling vigor under cold stress in US weedy rice. *Euphytica* 208:251-264. DOI 10.1007/s10681-015-1584-z

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