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COLD TOLERANCE OF AN INBRED LINE POPULATION OF RICE (*ORYZA SATIVA* L) AT DIFFERENT GROWTH STAGES

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ABSTRACT

Indica rice cultivar Hokuriku142, japonica rice cultivar Hyogokithanishiki and a recombinant inbred line population involving these cultivars were evaluated for cold tolerance at germination, post-germination and seedling stages. For each experiment 10 replicates were arranged with 20-40 seeds per replicate. At germination stage, cold stress was applied at 20°C and 15°C and the number of germinated seeds was counted. Post-germination stage cold tolerance was evaluated on 4-day germinated seeds byholding them at 4°C for 1-12 days and gained hypocotyl length was measured after a 4-day recovery period at normal growth conditions. To assess the seedling stage cold tolerance 1-week-old seedlings were maintained at 4°C for 1-7 days and green plant height was measured after a 5-day recovery period. In all growth stages Hyogokithanishiki showed higher degree of cold tolerance than Hokuriku. Recombinant inbred line population showed normal distribution curves for germination and post-germination stage cold tolearance with transgressive segregants for both higher and lower levels than the parents. At the seedling stage, the inbred populations showed a skew towards the susceptible cultivar but there were two transgressive segregants for greater cold tolerance than Hyogokithanishiki. Identification of such significant differences in the two parental rice cultivars and distribution of the character across the range of different tolerance levels with transgressive segregation indicates that this population is useful for the development of cold tolerant rice cultivars and to understand the basis of the cold regulation of rice using molecular tools.

Key words: Cold Tolerance, Rice

INTRODUCTION

Cold stress at the seedling stage and high temperatures at flowering stage occur in the regions of subtropics where rice is cultivated. In the temperate regions rice growth is constrained by the limited period that favors growth where it needs optimum temperature between 25°C to 35°C and temperatures below this often result in poor seedling vigor (Reyes *et al.* 2003).Temperatures below 25°C would cause growth abnormalities in temperate and high-elevated tropical rices (Fujino*et al.* 2004; Sthapit*et al.* 1998). Therefore development of rice cultivars with considerable level of cold tolerance is needed.

Continuous selection for high yield can result in the narrowing down of gene pool while it reaches a yield plateau. In Korea rice yield plateau has prevailed for twenty years regardless of many efforts by rice breeding programs (Moon *et al.* 1994). One way of overcoming this problem is to introgress *Indica* and *Japonica* gene pools (Dilday 1990). Genetic divergence studies among 17 rice varieties known to possess some degree of cold tolerance at different growth stages revealed that *Indica* group has moderate to low degree of cold tolerance while the *Japonica* group displayed high degree of cold tolerance (Khush *et al.* 1999). Kwon *et al.* (2002) demonstrated that Amplified Fragment Length Polymorphic (AFLP) markers are not useful for predicting heterosis for cold tolerance in *Japonica* hybrids but progenies of *Indica* and *Japonica* crosses provide genetic resources for development of cold tolerance of rice (Miura *et al.* 2001; Andaya and Mackill 2003a, 2003b; Saito *et al.*, 2004; Andaya and Tai 2006, 2007; Zhang *et al.* 2005a).

Conventional breeding is a slow process for generating crop varieties with improved tolerance to stress conditions (Wu and Ho 1999). Incompatibility in crosses between distantly related plant species and limited germplasm resources for stress tolerance are also barriers encountered in conventional breeding. Integration of compatible molecular biological methods with conventional breeding

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programs accelerates the process of varietal improvement of rice. Among those techniques, Quantitative Trait Loci (QTL) analysis is a useful tool to dissect responsive genes for desirable characters of populations with enough phenotypic variance.

QTL analysis of rice using progenies derived from crosses between *Indica* and *Japonica* cultivars has been performed by many researchers. Andaya and Tai (2006) used a population derived from *Japonica* rice cultivar M202 and *Indica* rice cultivar IR50. Lou *et al.* (2007) used a population derived from a cross between *Japonica* cultivar AAV 002863 and *Indica* cultivar Zhenshan 97B. A cross between *Indica* landrace cultivar Kunmingxiaobaigu and a *Japonica* rice cultivar Towada was used by Dai *et al.* (2004) for analysis of cold tolerant QTLs at booting stage. Fujino and Sekiguchi (2005) used *Japonica* crosses successfully to detect heading date QTLs in rice grown in northern limit for cold tolerant and long day-length responsive QTLs. Takeuchi *et al*. (2001) also used a *Japonica* X *Japonica* cross for QTL detection at booting stage. Other than *Japonica* X *Indica* crosses, some investigators used double haploid populations (Qian *et al.* 2000; Gao *et al.* 2007) and introgression lines (Saito *et al.*2004).

Cold tolerant QTLs at seed germination, vegetative and booting stages have also been identified in different populations (Fujino *et al.* 2004; Saito *et al.* 2001; Andaya and MacKill 2003a, 2003b; Andaya and Tai 2006 2007, Zhang *et al.* 2005a). Cold tolerant QTLs are different in different populations and are varied with the developmental stage in the same population of the plants as cold tolerance is developmentally regulated and growth stage-specific (Foolad 2001). The cold tolerance at one developmental stage is therefore not necessarily correlated with the tolerance at other stages (Foolad, 2001). It has been demonstrated that cold tolerance at the vegetative growth stage is independent of that at the reproductive growth stage of rice (Kaw *et al.* 1986). Thus, the development of methods for evaluation of cold tolerance for each developmental stage of rice life cycle need to be identified by careful examination of morphological changes in each developmental stage under the stress (Foolad 2001).

Better seedling establishment is very much responsible for the vigorous growth in vegetative stage with suitable green canopy, which is an advantageous character for higher starch production in later reproductive phase. The optimum temperatures for rice germination and early seedling growth were reported as 25-35.8^{o}C and the presence of some QTLs for cold tolerance at seedling stage of rice have been published by Yan *et al.* (1999), Andaya *et al.* (2002), Zhang *et al.* (2005a), Qian *et al.* (2000), Misawa *et al.* (2000), Lou *et al.* (2007) and Andaya and Tai (2007) in different populations.

Seedling vigour is a parameter to evaluate plant`s withstanding strength against any kind of stress conditions. Shoot length wasreported to be the best variable determinant ofseedling vigour (Peterson *et al.,*1978). Redo~na and Mackill (1996) found that shoot weight, shoot length and coleoptiles length were the best determinants to predict greenhouse and field seedling vigour of rice. In a study carried out by Zhang *et al.*(2005b)shootlength was the only character associated with all of the five seedling vigour QTLs identified at different temperatures considering shoot length, germination rate, root length and dry matter production of the inbred line population, thus providing strong support for the suggestion that shoot length is the best predictor of seedling vigour in rice. However they proposed that multiple related traits should be considered in experiments for any kind of QTL mapping, which will reveal genetic relationships among such complex traits. In the present study two *Japonica* and *Indica* parental rice cultivars were evaluated for cold tolerance at germination, post germination, and seedling stage and the methodologies adapted to evaluate these parents were applied to evaluate 163 inbred line population derived from these parents. The results showed the suitability of the population for QTL analysis at germination, post germination and seedling growth stages.

MATERIALS AND METHODS

A recombinant inbred line (RIL) population (F_6 generation) of 163 lines derived from a cross between cold tolerant *Japonica* rice cultivar Hyogokithanishiki, and a cold susceptible

Indica rice cultivar Hokuriku-142 was used. Hokuriku-142 (Hokuriku) was bred from a cross between a Korean cultivar, `Milyang 21` and an IRRI line ` IR-2061-214-31` in Hokuriku Agricultural Experimental station, Japan. Inbred line populations were advanced according to the single seed descent method and were selfed at each generation.

Germination stage cold tolerance
Dormancy broken surface sterilized seeds were maintained at 20^{o}C for two days and then temperature of the incubator was changed to 15^{o}C. Rate of germination of two cultivars were measured daily. Seeds those hypocotyl lengths were greater than the seed length; approximately 5mm were considered as germinated seeds. Considering the results of experiment with the two parental rice cultivars, initial two-day period at 20^{o}C and 12 days at 15^{o}C was applied to the inbred line population and the number of germinated seeds were counted at the end of the experiment.

Post germination stage cold tolerance
Dormancy broken, surface sterilized seeds of parent cultivars were germinated at 35^{o}C for 4 days. Cold stress was applied at 4^{o}C for 12 days to 4-day-oldgerminating seedlings, followed by a 4-day recovery period at 25^{o}C. Gained shoot length during the cold stress and the recovery period was used as a parameter for evaluating the level of cold tolerance (Fig.1).For the RIL population, a 5-day cold treatment at 4^{o}Cwas applied for the evaluation of cold tolerance at post germination stage. Twenty seeds of each line were subjected to cold tresses in separate experiments and experiment was repeated three times. Experiment was arranged according to a complete randomized design and data was recorded on the 5th day of the recovery period followed by the cold stress.

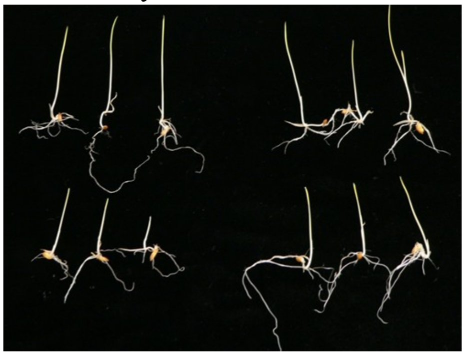

Figure. 1 Germinated seedlings of two parental rice cultivars under control condition and under cold stress at post germination stage (HOK -Hokuriku-142, HGKN – Hyogokithanishiki)

Seedling stage cold tolerance
Breakage of dormancy and uniform germination were achieved by maintaining surface-sterilized seeds at 35^{o}C for 6 days in distilled water. On the 6th day, germinating seeds were planted in Petridishes and maintained at 25^{o}C for one week under a 16hour photoperiod (normal growth conditions). On the 7th day, seedlings (13-day -old) were transferred to 4^{o}C for cold stress and maintained up to 7 days. Green shoot length was measured on the 5th day of the recovery period under normal growth conditions and the survival rates of the two cultivars under cold stress at seedling stage were calculated.

Based on the results of parent cultivars, 3-day cold stress at 4˚C was used for scoring the cold tolerance in RILs. The level of cold tolerance was evaluated according to a five-point rating scale (Fig. 3) on the 5th day of the recovery period.

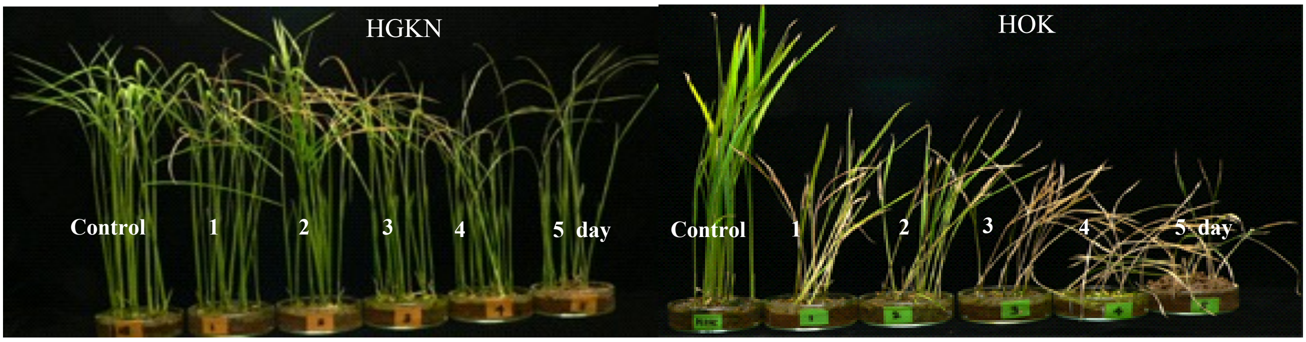

Figure 2 Two rice cultivars in recovery period, stressed at 4^{o}C cold stress for 1: 1-day,2: 2-day, 3:3-day, 4: 4-day, 5: 5-day cold stress period (HOK - Hokuriku-142, HGKN – Hyogokithanishiki)

Figure 3 Five point rating scale used for the evaluation of inbred line population.1- the whole seedling became completely withered, 2- 1st and 2nd leaves became withered but the stem remained green, 3 - only the stem and 3rd leaf remained green, 4 – the stem and two leaves remained green, and 5 - normal growth with all the leaves remained

Analysis of correlation

Pair wise correlation analysis was performed to understand the relationship in cold tolerance at different growth stages in rice.

RESULTSAND DISCUSSION

Germination stage cold tolerance

In the present study seeds started germination at20^{o}C,while at 15^{o}C seed germination was retarded. With time, seeds of Hyogokithanishiki continued germination while those of Hokuriku stopped further hypocotyls protrusion. On the 12th day at 15^{o}C, a significant difference in germination percentage between the two parental rice cultivars could be seen. Hokuriku recorded 0-8% germination at low temperature and its average germination at low temperature was 2.6%. Hyogokithanishiki recorded 66.7-91.6% germination at low temperature, with an average of 77.3% (Fig.4). Most studies performed at germination stage in rice under controlled temperature to identify the variability and genotype characterization showed that genotypes belonging to *Japonica* subspecies are more cold tolerant than *Indica* (Li *et al.,* 1981; Cruz & Milach, 2004). The reason for this difference would be that the *Japonica* rice cultivars evolved in temperate regions while the evolution of *Indica* rice cultivars has taken place in tropical climates. At the early seedling stage and seedling stage *Japonica* was better than *Javonica, Indica* and wild species while at panicle development and flowering stages*Javonica* was better than the other types (Visperas and Vergara, 1981). The authors showed not only the varietal difference in cold tolerance on basis of origin but also the stage of growth of the plant.

Inbred line population showed normal distribution for low temperature germination and many lines recorded higher germination percentage at low temperature than that of Hyogokithanishiki (Fig. 4). Transgressive segregants were recorded in both directions showing that both parents possess cold tolerant alleles and therefore this population can be easily used for QTL analysis in rice cold tolerance at germination stage.

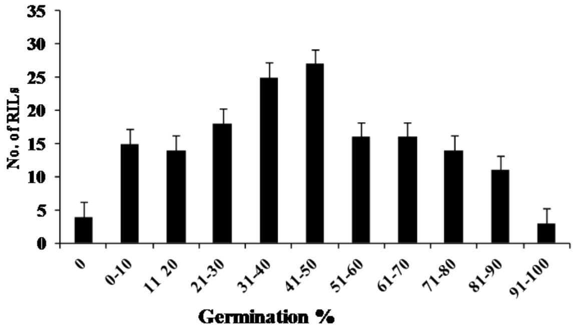

Figure 4. Population distribution of low temperature germination rate in the recombinant inbred population and parent cultivars. Two-day cold period at 20^{o}C followed by 12 days at 15^{o}C was applied to evaluate inbred line population for cold tolerance. Bars indicate standard deviations. HOK - Hokuriku-142, HGKN – Hyogokithanishiki

Evaluation of populations for low temperature germinability has been widely practiced in different populations and different low temperature stresses for different durations such as 15^{o}C for seven-days (Fujino 2004), 15^{o}C for four-days (Miura *et al.* 2001), 15^{o}C for 6 days (Liang *et al.* 2006), 13^{o}C for 28 days (Cruz *et al.* 2004), 14^{o}C for7-17days (Zhi *et al*. 2006), 13^{o}C for 30 days (Cruz *et al.* 2006) and 17^{o}C for 7 days (Stapit and Witcombe, 1998) have been used. This procedure has not been standerdised, and needs to be adjusted for each population. Optimum temperature range for rice germination lies between 20-35°C, and the temperature of 10°C is cited as the minimum critical value below which rice does not germinate (Yoshida 1981b). Seeds were submitted to temperatures varying from 10 to 25°C for periods of three to thirty five days and parameters such as germination percentage, rate of germination, and coleoptile and radicule length (Maya 1988; Srinivasulu & Vergara 1988; Bertin *et al.* 1996; Sthapit & Witcombe 1998) have been used in cold tolerant assessment at germination stage.

Germination is divided into three phases: imbibition, activation and post- germination growth (Yoshida 1981a). The greatest effect of cold temperature during germination has been attributed to the imbibition phase, considered to be the most sensitive (Cruz et al., 2004). In the present study, 20°C, a comparatively mild cold stress was given at the initial stage of germination and a moderatelysevere15°C cold stress was given at activation period. However, Yoshida (1981b) reports that the greatest influence of temperature on germination occurs in the subsequent phases of activation and growth of coleoptile and radicule. However, reports on the effect of temperature on the germination period of rice is contradictory and the existence of different physiological mechanisms involved with cold tolerance at the vegetative period is an indication that even at the germination phases, different processes may also be affected implying in different tolerance mechanisms. Massardo *et al.* (2000) demonstrated that higher metabolic rates and less oxidative damage were the reason for the tolerance of genotypes to cold temperature during germination in Oat.

Post-germination stage cold tolerance

In our bioassay HOK showed a higher shoot length gain until the 4th day of cold stress than HGKN, whereas after the 5thday of cold stress, HGKN recorded a greater value than HOK (Fig.5). This would be the reason that HGKN acquired cold acclimation by the 5th day of cold treatment while HOK failed. Increase in freezing tolerance upon exposure to low non-freezing temperatures is called as cold acclimation (Thomashow 1999). Kim and Tai (2011) also showed that some *Indicas* provide useful genes for improving seedling cold tolerance in *Japonicas*.
After the 5th day of cold stress, shoot gain decreased in both parents, but *Japonica* parent, HGKN maintained higher mean values until the end of the experiment, i.e. 12-day cold stress. This can be explained by the fact that *Japonica* cultivars, which are grown in both tropical and temperate zones, are typically more cold tolerant than *Indica* cultivars, which are found mostly in tropical lowland areas (Glaszmann *et al.* 1990; Mackill and Lei1997; Baruah *et al.* 2009).

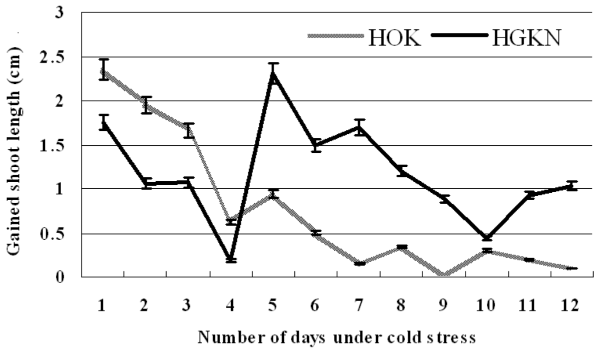

Figure 5. Gained shoot length of two parental rice cultivars after different days of cold stress at 4°C and recovered at 35°C in the dark for 4-days at post germination stage. The values are averages of three replicates and each replicate consisted of twenty-seeds. HOK - Hokuriku-142, HGKN – Hyogokithanishiki

Populations were studied for post germination stage or early seedling stage cold tolerance by Zhang *et al.* (2005a) and Yan *et al.* (1999) using different conditions than what we applied. Specific procedures for the particular population need to be selected through series of preliminary studies for the better exploration of the population specific characters.

The frequency distribution of RILs showed9 different classes based on the gained shoot length after 5-day cold stress (Fig.6). The gained shoot length in RILs showed a nearly normal distribution, indicating that this trait is under polygenic control. A number of transgressive segregants showing either smaller or larger values than those of the parents were observed after the 5-day cold stress emphasizing the contribution of both parents to cold tolerance of inbred line population. More vigorous low temperature germinability than the recurrent parent was recorded in several studies (Fujino*et al. 2008*; Iwata and Fujino 2010).

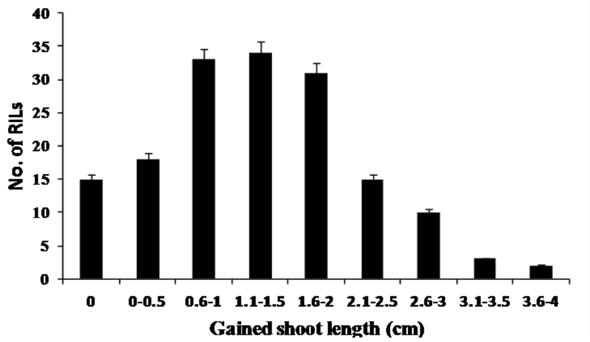

Figure 6. Frequency distribution of inbred line population according to the average gained shoot length after cold stress at 4°C for 5-days and recovered at 35°C in the dark for 4days. Bars indicate standard deviations. HOK - Hokuriku-142, HGKN – Hyogokithanishiki

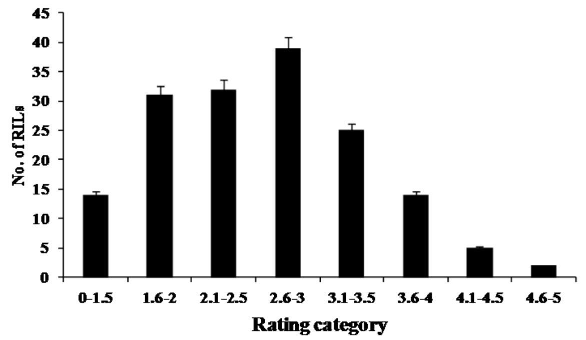

Figure 7. Frequency distribution of cold tolerance ratingsat the post-germination stage of the inbred line population after the cold treatment. The ratings range from 5 (cold tolerant) to 1 (susceptible). Five point rating scale used for the evaluation of inbred line population is given in Fig.3.Parent cultivars: HOK - Hokuriku-142, HGKN – Hyogokithanishiki.

Seedling stage cold tolerance

Visual assessment of morphological characters following direct exposure to low temperatures is the most common method of evaluation of cold tolerance at the seedling stage (Bertin *et al.* 1996). Cold tolerance at the seedling stage in rice has mainly been studied at temperatures ranging from 4 to 10°C (Qian *et al.* 2000; Andaya and Mackill 2003a; Zhang *et al.* 2005a, Misawa *et al.* 2000). In each case different bio assays have been applied and different indices were evaluated such as leaf chlorosis/discoloration (Kwak *et al.* 1984; Andaya and Mackill 2003a; Han *et al.* 2004), withering, necrosis, mortality (Nagamine 1991; Andaya and Mackill 2003a; Lou *et al.* 2007), reduced seedling vigor (Zhang *et al.* 2005b), and decreased recovery of shoots (Baruah *et al.* 2009). In the present study length of green shoot was considered as the parameter for the estimation of cold tolerance.

All seedlings of the *Indica* cultivar HOK turned brown and died at the 5th day of recovery period after 3 days of cold stress, whereas all seedlings of the *Japonica* cultivar HGKN retained green shoots with their mean length of 17.4cm under the same stress condition (Fig.7). The results showed a significant difference in the two cultivars for cold tolerance at the seedling stage from 3 day cold stress onward(Fig.7). To evaluate the inbred line population derived from these two parents, an arbitrary rating scale was developed considering the green shoot length of the recovered seedlings (Fig. 3)

For post-germination stage cold tolerance RILs showed a little left-skewed distribution towards cold susceptibility (Fig. 7, Fig.3). HGKN showed an average rating of 4.4, while that of HOK was 1.5 (Fig. 7). Two RILs were showed higher level of cold tolerance than the cold tolerant parent HGKN.

Analysis of variance demonstrated significant variability in ratings between the lines but no significant differences within the line, indicating that seedling response to the cold treatment was homogeneous within each line.

Correlation analysis between growth stage and cold tolerance showed a positive but weaklysignificant (at 0.0145 significance level) correlation (correlation coefficient=0.1934) between post germination and seedling stage but there was no correlation between other growth stages. Li *et al.* (1981) also reported a significant positive correlation in cold tolerance between early seedling stage and seedling stage in rice. This may indicate the existence of similar cold tolerance mechanisms in early seedling stage and seedling stage but existence of different cold tolerance mechanisms in cold tolerance at germination stage.

CONCLUSION

For the evaluation of germination stage cold tolerance, 2-day cold stress at 20°C followed by a 12-day cold stress at 15°C and continuously in dark condition is appropriate for a population derived from Hyogokithanishiki and Hokuriku.

A cold stress for 5 days at 4°C for 4-day germinated seedlings at 35°C is suitable for the evaluation of post-germination stage cold tolerance and 3-day cold stress at 4˚C followed by a 5 day recovery period is suitable for scoring the level of cold toleranceat the seedling stage.

This protocol may be applied to a larger set of rice accessions in identifying useful materials for breeding and genetic studies of germination, post-germination and seedling cold tolerance.

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