

DEVELOPMENT OF A METHOD FOR TESTING DEHYDRATION TOLERANCE IN RICE (*ORYZA SATIVA* L) AND ITS APPLICATION TO A RECOMBINANT INBRED LINE POPULATION

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ABSTRACT

A recombinant inbred line population derived from *Japonica* rice cultivar Hygokithanishiki and *Indica* rice cultivar Hokuriku-142 is a valuable source for genetic and molecular studies as these two parents have different origins. Characterization of the two parents under different biotic and abiotic stress conditions exploits novel research areas such as QTL analysis. In the present study two parents were evaluated for dehydration tolerance using Petri dish method and nursery box method. Average root length, shoot length, root dry matter and shoot dry matter weight were evaluated in each method. Shoot length in nursery box method gave a significant difference between two cultivars with Hygokithanishiki dehydration tolerant and Hokuriku-142 dehydration susceptible. The method was applied to evaluate 163 recombinant inbred lines (RILs) derived from the two parents for dehydration tolerance. The trait showed a normal distribution in the population with some transgressive lines expressing greater dehydration tolerance than dehydration tolerant parent Hygokithanishiki. Therefore nursery box method is proposed as a reliable method for the evaluation of dehydration tolerance of rice cultivars and this method is suitable for evaluating germplasm collections and breeding populations for dehydration tolerance in rice breeding.

Key words: Rice, Bioassay, Breeding, Dehydration Tolerance, Drought Resistance

INTRODUCTION

Drought is the single greatest factor limiting rice production in approximately 46 million ha of Asia where rice is the main food (Pandey 2000). The conventional breeding techniques have recently been integrated with more efficient phenotyping involving molecular markers and genomic technologies to improve drought tolerance by exploitation of the broad genetic diversity in the primary gene pool of rice (Lafitte *et al.* 2004). There are two options for the management of crops in water limiting environments: the agronomic and the genetic management. Development of this genetic management technology requires robust, reproducible, simple, and rapid field, pot, and laboratory screening methods for identification of traits of drought tolerance in germplasm, and incorporation of the same in high-yielding varieties using conventional and biotechnological tools.

Drought tolerance is considered to be a difficult topic as it is not possible to define or measure tolerance with the same clarity or precision as it is for disease resistance or for mor-

phological or other physiological traits, nor is it easy to manage experimental drought environments with a high level of control and repeatability. Therefore developing a functional definition of drought tolerance to use in screening programs, designing screening procedures to focus effectively on the target environment, and managing the screening experiments to increase precision in detecting heritable differences in tolerance are repeatedly studied.

The reason of the slow progress in developing drought tolerant rice has been identified as the lack of a specific method for screening the large numbers of genotypes required in breeding for drought (Zeigler and Puckridge, 1995). Phenotyping protocols differ among drought tolerant screening and scoring studies. Many traits, such as root traits (Sun *et al.* 1995; Champoux, *et al.* 1995) leaf characters (Champoux *et al.* 1995; Mitchell *et al.* 1998), proline accumulation (Yang *et al.* 1995), membrane stability and osmotic adjustment (Li *et al.* 2005), shoot height (Wang *et al.* 2005), biomass and panicle characters (Lanceras *et al.* 2004; Yue *et al.*, 2005) have been considered as drought scoring traits in rice. Other than the-

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se, grain yield (Wang *et al.* 2005), yield components (Lanceras *et al.* 2004; Yue *et al.* 2005; Yue *et al.* 2008) and harvest index (Babu *et al.*, 2003), have also been scored to evaluate drought tolerance in rice. In an upland rice breeding program in Brazil, secondary traits of low leaf rolling, good panicle exertion, and low level of spikelet sterility have been considered as selection criteria (Pinheiro and Da 2006) for drought tolerance. In root phenotyping, thickness of roots measured at basal or deeper soil layers (Kamoshita *et al.* 2002a), deepness of roots measured as maximum rooting length (Hemamalini *et al.* 2000), seminal or adventitious root length (Zheng *et al.* 2003), or total or deep root dry weight (Yadav *et al.* 1997) had been reported. However, accurate field phenotyping of mapping populations for drought tolerance has remained complex and difficult. The effectiveness of a drought screening procedure is best measured by the genetic heritabilities achieved for target traits, whether the focus is conventional or marker-assisted plant breeding.

Accurate phenotyping in the initial molecular mapping is the most important pre-requisite to success in selecting for drought resistance. Phenotyping is often said to be inadequate owing to poor measurement techniques, irrelevant manipulation of experimental drought conditions, lack of reliable plant and soil data, and lack of field experts to interpret the results of experiments on plant function under drought stress (Blum *et al.* 2005). Plant height and flowering time usually are highly heritable and are extensively used in traditional plant breeding (Cooper *et al.* 1999a). Leaf rolling and canopy temperature (Lafitte *et al.* 2004; Hirayama *et al.* 2006), root characters (Zhang *et al.* 1999, Zhang *et al.* 2001) are also studied for quick screening of hundreds of lines. Measurement of induced traits such as osmotic adjustment or cell membrane stability could involve greater errors than measurement of constitutive traits because of differences in the degree of water stress if experimental conditions are not precisely controlled.

Most putative drought-resistance traits considered for rice have low heritability and are not consistently correlated with grain yield under drought conditions in the target environments because of the large genetic and environment interactions in rainfed rice as well as the large

error variance under dry conditions (Atlin and Lafitte, 2002).

Many different drought tolerant screening or scoring techniques have been reported such as, keeping seedlings in two and half leaf stage in 30 mL PEG (PEG-6000) solutions in tubes (Zhou *et al.* 2006), growing seedlings in glasshouse to the 3-leaf stage and then placing in a growth chamber with programmed diurnal changes in light, temperature, and relative humidity followed by a 10-day exposure to growth chamber conditions without irrigation under five widely different environments (O'Toole *et al.*, 1978), comparison in irrigated lowlands and severely water stressed under managed stress conditions in uplands (Atlin *et al.* 2006), and growing during the dry season (Babu *et al.* 2003; Lanceras *et al.* 2004; Lafitte *et al.* 2004; Wang *et al.* 2005; Yue *et al.* 2005). Several studies have been conducted under both well-watered and drought-stressed conditions (Zou *et al.* 2005; Kumar *et al.* 2007), and some studies applied multiple stress regimes (Lanceras *et al.* 2004; Jearakongman, 2005). Rainout shelters can reduce damage from untimely rainfall and in China shelters were used with drip irrigation and drainage systems (O'Toole, 2004; Li *et al.* 2005; Yue *et al.* 2005; Liu *et al.* 2008). However, the high cost of such facilities may limit their widespread use. Raised beds, 30cm above ground level to screen drought-resistant genotypes (Hirayama and Suga, 1996) were also practiced in Japan. Because of independent domestication events for *Indica* and *Japonica* subspecies, a broad range of variation of characters within *indica* and *japonica* varieties can be expected (Gao *et al.* 2005). Drought tolerant inbred lines have been identified in different germplasm accessions (Yu *et al.* 2003; Ali *et al.* 2004; Lafitte *et al.* 2004) as new breeding materials for development of drought resistance in rice. In the present study a convenient and reliable method for the evaluation of individuals for drought tolerance was developed and the methodology was applied to evaluate an inbred line population of 163 lines derived from a *Japonica* and *Indica* cross to assess the feasibility of the method.

MATERIALS AND METHODS

A cold tolerant *japonica* rice cultivar Hy-

ogokithanishiki used in sake brewing, a cold susceptible *Indica* rice cultivar Hokuriku-142 and a recombinant inbred line (RIL) population (F_6 generation) of 163 lines derived from them were used in the study. Hokuriku-142 (Hokuriku) was bred from a cross between a Korean cultivar, 'Milyang 21' and an IRRI line 'IR-2061-214-31' at the Hokuriku Agricultural Experimental Station in Japan. High amylose content and low fatty acid content of Hokuriku-142 are favorable characters for better sake brewing in Japan and low fatty acid content causes high sensitivity to low temperatures. The initial cross was done for the development of sake brewing rice and present study was carried out using the same materials after advancing the populations up to F_6 generation. Inbred line populations were advanced according to the single seed descent method and were selfed at each generation. The parental rice cultivars were used as controls

For the Petri dish method, seeds of the parental cultivars were surface sterilized by dipping in 70% ethyl alcohol for 2 minutes and subsequently keeping seeds in 1% sodium hypochlorite solution for one hour. Seeds were germinated in water at 35°C for one week in the dark. Germinated seeds were planted in soil filled Petri dishes and allowed to grow for another one-week. During this period Petri dishes were dipped in a basin of 1ppm Hyponex solution (Toyoba, Japan) and the level of Hyponex solution in the basin was maintained by adding water daily. After one week, Petri dishes with plants were removed out of water (Fig. 1A) and left for drying. After 2 days plants were entirely dried and on the third day (one day after drying) four replicates of each parental line were returned to Hyponex solution for recovery. Another set of 4 replicates was returned to Hyponex solution on the next day (two days after drying) and the other set of four replicates were returned to Hyponex solution on the third day after drying. Plants were allowed to recover in Hyponex solution for a 10-day period (Fig. 1B). Length of green shoot, shoot dry matter weight and root dry matter weight of plants were scored at the end of the experiment.

In nursery box method, seeds of parental cultivars were surface sterilized and germinated as described above were planted in nursery boxes (15cm X 15cm X 7.5cm) so that one replicate

of two parental cultivars were in one nursery box side by side. Nursery boxes were kept in a basin of water with 1ppm Hyponex solution and plants were allowed to grow there for two weeks (Fig. 2A). After two weeks, nursery boxes were taken out of the basin and plants were allowed to dry (Fig. 2B). Plants were returned to the basin of water for recovery on the 1st, 2nd, 3rd, 4th and 5th day after the drying period. After 10 days of recovery (Fig. 2C, Fig. 2D), length of green shoot, length of root system (the longest root) and dry matter weight of plants were evaluated.

Considering the results of parental screening, 5-day drying period in nursery boxes was applied for the evaluation of the inbred line population. Fig.3 shows the bioassay conditions for evaluation of inbred line population derived from Hyogokithanishiki and Hokuriku-142 for dehydration tolerance.

Experiment was carried out according to complete randomized design and repeated three times. Average green shoot length was used as the parameter to evaluate the inbred line population on the 10th day of the recovery period after 5-day dehydration stress. Experiment was carried out in a growth chamber under control environment condition (25°C, 16h photoperiod). Data was analyzed using ANOVA.

RESULTS

Petri dish method

All plants of Hyogokithanishiki that were subjected to one day drying period recovered and turned green during the recovery period while only few plants of Hokuriku-142 recovered and turned green during recovery period. All the Hokuriku-142 plants that were subjected to dehydration stress for two days dried completely and did not recover after returning to water (Fig. 1).

Average green shoot length of the two parental rice cultivars showed almost the same value under the control conditions in Petri dish method. On the first day of dehydration stress, two parental cultivars showed much difference in phenotypic characters and by the second day of the stress the difference was quite prominent. Root length of the seedlings was not measured in this experiment as soil depth and experiment

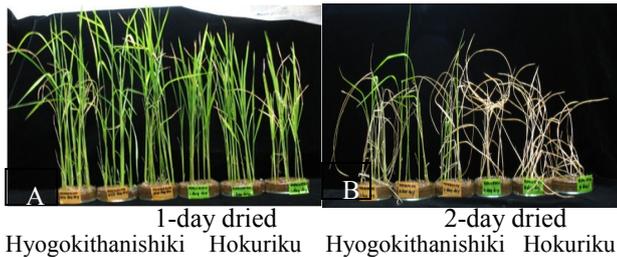


Figure 1 Effect of dehydration stress on two parental rice cultivars grown in Petri dishes. Hyogokithanishiki: *Japonica* rice cultivar, Hokuriku: *Indica* rice cultivar Hokuriku-142. A - One day after completely dried and recovered B - Two days after completely dried and recovered

duration was not sufficient for a proper assessment.

Under control conditions and under each dehydration stress condition, Hyogokithanishiki gained higher dry matter weight compared to that of Hokuriku-142. However, none of the parameter was significantly different between two stress regimes in the Petri dish method (Table 1).

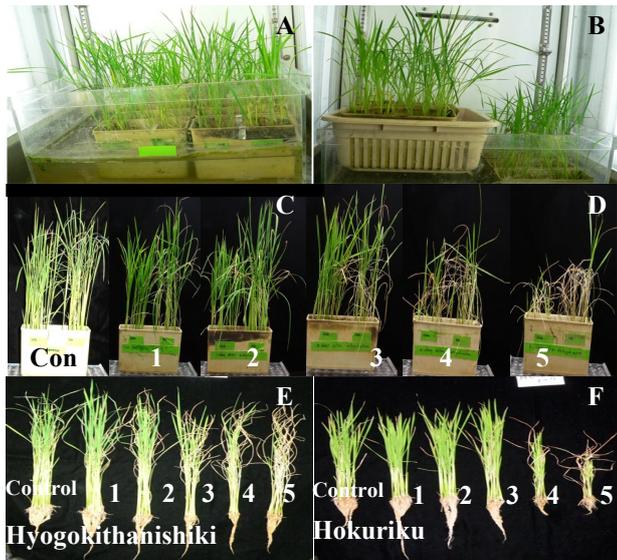


Figure 2 Comparison of two parental rice cultivars subjected to different days of dehydration stress. A. Plants under control condition B. Plants under dehydration condition C. Recovered plants after dehydration stress: Control (on left), 1-One-day dehydration, 2-Two-day dehydration, 3-Three-day dehydration, 4-Four-day dehydration, 5-Five-day dehydration. In each nursery box plants on the left are Hokuriku-142 while plants on the right are Hyogokithanishiki D. Uprooted Hyogokithanishiki plants after 1 to 5 days of dehydration stress compared with the control E. Uprooted Hokuriku-142 plants after 1 to 5 days of dehydration stress compared with control plants

Table 1 The level of significance in each parameter.

Method	Parameter	P value
Petri dish method	Green shoot length	0.406942
	Shoot dry matter	0.43433
	Root dry matter	0.390652
	Shoot length	0.058062
Nursery box method	Root length	0.214967
	Shoot dry matter	0.155056
	Root dry matter	0.366174

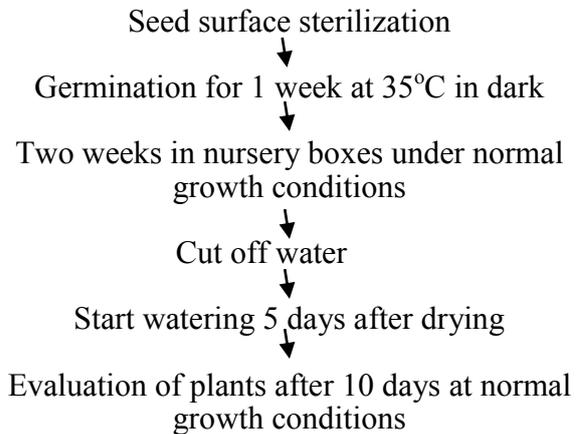


Figure 3 Bioassay conditions for evaluation of inbred line population for dehydration tolerance.

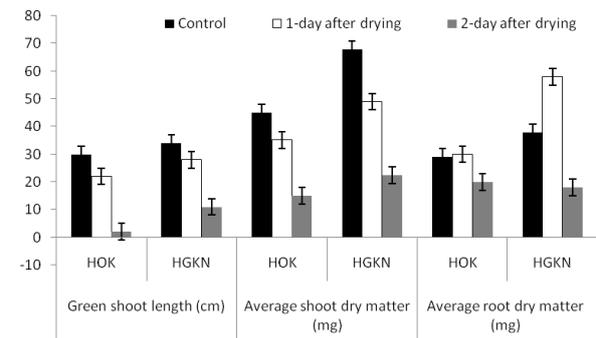


Figure 4 Green shoots length, shoot dry matter weight and root dry matter weight of two parental rice cultivars after dehydration stress in Petri dish method. HGKN: Hyogokithanishiki, HOK: Hokuriku-142. Data was taken at the 10th day of the recovery period after 1 and 2 days of drying of the seedlings. Standard errors are indicated in bars.

Among parameters evaluated in nursery box method, green shoot length of parental rice cultivars was significantly different at 0.05 probability level (Table 1).

Evaluation of inbred line population for dehydration tolerance

The inbred line population was evaluated for drought tolerance by assessing green plant height after a 10 day recovery period following 5 days of drying. The trait showed nearly normal distribution with a skew towards susceptibility (Fig. 5).

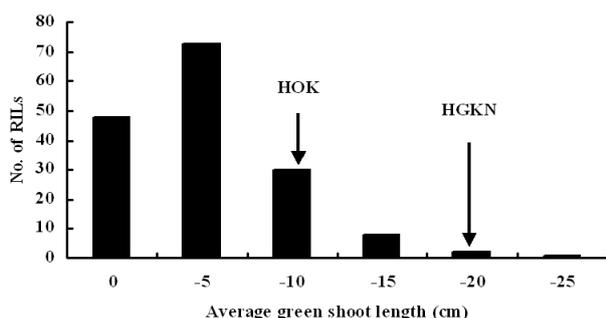


Figure 5 Frequency distribution of the inbred line population for green shoot length after a 5-day dehydration period. Data were recorded on the 10th day of recovery period after a 5-day dehydration stress. Twenty seedlings of each parent and inbred line were subjected to dehydration stress and the experiment was repeated three times.

DISCUSSION

For efficient screening of dehydration tolerance, Blum (2002) suggested consideration of some aspects of assessing the utility of traits. One was that important drought resistance traits are normally constitutive and not stress adaptive. Constitutive traits, such as flowering time, stay-green trait (delayed onset of leaf senescence), and root depth, were recommended to assess in routine screenings (Blum 2002). Deep and thick root traits contribute to better growth and higher yield under drought stress (Lafitte and Courtois 2002; Babu *et al.* 2003). However in the present study root length was not studied in Petri dish method, and in nursery box method, root length was not significantly differed between cold tolerant and cold susceptible drought stressed plants. This short period of stress and limited soil depth would not create such difference in two cultivars. Hokuriku-142 recorded longer roots compared to Hyogokithanishiki under control conditions (Fig.2D and 2E). Until 3-day dehydration period, Hokuriku-142 recorded longer roots, but from the third day of dehydration, Hyogokithanishiki recorded longer roots. At the fifth day of dehydration, Hyogokithanishiki recorded significantly longer roots. The reason would be that, at initial drought stress period, root system of Hokuriku-142 would respond in its maximum capacity while under continued stress conditions Hokuriku-142 failed to survive, but Hyogokithanishiki performed better because of its greater tolerance to severe drought stress conditions. Thus, Hokuriku-142 performed better at initial drought stress period

than Hyogokithanishiki and Hyogokithanishiki performed better at later stages of drought stress than Hokuriku-142. Overall, analysis of variance of root length did not reveal a significant difference between the two cultivars. This indicates that neither parent carries all of the positive or negative alleles for drought resistance. This is supported by grain yield under drought stress where positive alleles contributed by both parents, as evidenced by transgressive segregation under drought stress (Babu *et al.* 2003; Lanceras *et al.* 2004; Xu *et al.* 2005).

The role of drought adaptive traits towards drought resistance is considered to be greater than stress responsive traits such as active cellular accumulation of compatible solutes (osmoprotectants), antioxidant agents, heat shock proteins, and molecular chaperones, as well as osmotic adjustment and membrane stability (Kamoshita *et al.* 2008). Plant-type traits (e.g., plant height) and phenology (e.g., flowering time) usually are highly heritable and are extensively used in traditional plant breeding (Cooper *et al.* 1999a, b). In the present study green shoot length was considered as one of the parameters for evaluation of dehydration tolerance. Hyogokithanishiki recorded higher green shoot length under drought stress (Fig.2D and 2E) compared to Hokuriku. Hence, green shoot length under dehydration can be considered as a useful trait for screening a population derived from the parents used in this study. Constitutive root traits, interacted with drought intensity, have a large effect on extractable soil water during drought (Lilley and Fukai, 1994). This influences expression of both induced and secondary traits such as maintenance of plant water status, canopy temperature, leaf rolling score, and leaf death score (Lilley and Fukai 1994). High dry matter weight of root system of Hyogokithanishiki under severe drought stress is additional evidence for higher dehydration tolerance of Hyogokithanishiki than Hokuriku-142. Umayal *et al.* (2001) observed that drought tolerant *Indica* landraces in southern India had thicker roots with wider xylem vessels. Deeper and thicker roots also may occur under upland conditions and some lowland conditions, helping the plant to absorb larger amounts of soil water, thus maintaining higher plant water status (Yoshida and Hasegawa, 1982; Kumar *et al.* 2004) Average root dry matter weight of Hy-

ogokithanishiki increased drastically by the first day of dehydration stress compared to control plants but by the second day of dehydration stress both cultivars showed sudden decrease in root dry matter (Fig.4). However, high dry matter accumulation in the root system on the first day of dehydration stress in Hyogokithanishiki indicates its better dehydration tolerance response. However, the non-significant difference in root dry matter production of drought tolerant rice cultivars under drought stress has also been reported (Ekanayake *et al.* 1985; Samson *et al.* 2002; Kamoshita *et al.* 2002a; Pantuwan *et al.*, 2004; Yue *et al.* 2006; Kumar *et al.* 2007). The measurements of root traits under drought conditions in the field usually have large errors (Pantuwan *et al.* 2004; Samson *et al.* 2002), and the broad-sense heritabilities of root traits measured in the field (e.g., Kumar *et al.* 2007) are in general lower than those measured under hydroponic systems (Ekanayake *et al.* 1985) and pot systems (Kamoshita *et al.* 2002a; Yue *et al.*, 2006). Pantuwan *et al.* (2004) reported that root responses to dry conditions among genotypes in the dry season differed from responses under wet season drought. So, the root trait results of any kind of drought screening under control environmental conditions may deviate more or less from the reality.

Among measured parameters in the present study, only green shoot length of nursery box method was significantly different in two parents at 0.05 probability level (Table 1). Green shoot length of survived rice seedlings on the 10th day of recovery period after 5 days of drying stress on two-week-old seedlings was applied to evaluate dehydration tolerance in inbred line population derived from the two parents used in this study; *Japonica* rice cultivar Hyogokithanishiki and *Indica* rice cultivar Hokuriku-142.

The phenotypic distribution in the inbred lines for green shoot length did not show discrete classes but approximately fitted a normal distribution, indicating that green shoot length is quantitatively inherited in nature. Transgressive segregation in both directions was observed for the trait (Fig.5) under 5 day dehydration stress, indicating that both parents transmitted favorable alleles for the trait. The population used in the present study possessed

3 inbred lines with longer green shoot length than drought tolerant Hyogokithanishiki (Fig.5). Phenotypic frequency distributions support the quantitative inheritance of drought tolerance genes. This method can be successfully applied for large scale screening for drought tolerant at seedling stage in rice.

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