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QTLs for cell-membrane stability mapped in rice (*Oryza sativa* L.) under drought stress

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Abstract Cell-membrane stability (CMS) is considered to be one of the major selection indices of drought tolerance in cereals. In order to determine which genomic region is responsible for CMS, 104 rice (*Oryza sativa L.)* doubled haploid (DH) lines derived from a cross between CT9993–5-10–1-M and IR62266-42–6-2 were studied in the greenhouse in a slowly developed drought stress environment. Drought stress was induced on 50-day-old plants by withholding water. The intensity of stress was assessed daily by visual scoring of leaf wilting and by measuring leaf relative water content (RWC). The leaf samples were collected from both control (well-watered) and stressed plants (at 60–65% of RWC), and the standard test for CMS was carried out in the laboratory. There was no significant difference ($P>0.05$) in RWC between the two parental lines as well as among the 104 lines, indicating that all the plants were sampled at a uniform stress level. However, a significant difference ($P<0.05$) in CMS was observed between the two parental lines and among the population. No significant correlation was found between CMS and RWC, indicating that the variation in CMS was genotypic in nature. The continuous distribution of CMS and its broad-sense heritability (34%) indicates that CMS should be polygenic in nature. A linkage map of this population comprising of 145 RFLPs, 153 AFLPs and 17 microsatellite markers was used for QTL analysis. Composite interval mapping identified nine putative QTLs for CMS located on chromosomes 1, 3, 7, 8, 9, 11 and 12. The amount of phenotypic variation that was explained by individual QTLs ranged from 13.4% to 42.1%. Four significant ($P<0.05$) pairs of digenic interactions between the detected QTLs for CMS were observed. The identification of QTLs for this important trait will be useful in breeding for the improvement of drought tolerance in rice. This is the first report of mapping QTLs associated with CMS under a natural water stress condition in any crop plants.

Key words Cell membrane stability · Drought resistance · *Oryza sativa* · QTLs · Rice

Introduction

Drought is the major abiotic stress limiting rice yields in rainfed environment (Toenniessen 1991). Tolerance to this abiotic stress is a complex phenomenon, comprising of a number of physio-biochemical processes at both cellular and organismic levels at different stages of plant development. One of the options to overcome this problem is to dissect the complex traits into different components that are highly heritable, easy to measure and repeatable.

There are two major strategies of drought resistance: dehydration avoidance and dehydration tolerance (Levitt 1980). The avoidance mechanism helps plants to maintain relatively high leaf water potential during drought by extracting more water from the soil through deep roots (O'Toole and Bland 1987; Ludlow 1989) or by reducing water loss through closed stomata and leaf movement (Turner 1979; Ludlow and Muchow 1990). Rice lines with a large system of deep roots tend to have high leaf water potential and delayed leaf death during drought (Mambani and Lal 1983; Cruz and O'Toole 1985). The tolerance mechanism helps plants to maintain turgor and volume, thus continue metabolism even at a low leaf water potential (Turner and Jones 1980; Morgan

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1984; Nguyen et al. 1997). Dehydration tolerance in rice may allow the plant to maintain the metabolic activity for longer periods and to translocate more stored assimilate to the grain (Fukai and Cooper 1995). The strategy adopted by the plant for tolerance to water stress involves a high desiccation tolerance through osmotic adjustment (OA) and maintenance of cell-membrane stability (CMS).

The cell membrane is one of the main cellular targets common to different stresses (Levitt 1980). The extent of its damage is commonly used as a measure of tolerance to various stresses in plants such as freezing (Dexter 1956), heat, drought (Blum and Ebercon 1981) and salt (Leopold and Willing 1983). Bewley (1979) reviewed the desiccation tolerance in higher plants and concluded that the critical features of desiccation tolerance depend on the abilities to limit membrane damage during water stress and to regain membrane integrity and membrane-bound activities quickly upon rehydration.

Cell-membrane stability or the reciprocal of cell-membrane injury is a physiological index widely used for the evaluation of drought and temperature tolerance (Sullivan 1972; Martineau et al. 1979a; Blum and Ebercon 1981; Sadalla et al. 1990a; Reynolds et al. 1994; Fokar et al. 1998a). This method was developed for a drought and heat tolerance assay in sorghum and measures the amount of electrolyte leakage from leaf segments (Sullivan 1972). Its reliability as an index of heat stress tolerance is supported in several plant species by a good correlation between CMS and plant performance in the field under high temperature stress (Martineau et al.1979a; Sullivan and Ross 1979; Shanahan et al.1990; Sadalla et al. 1990b; Reynolds et al. 1994; Fokar et al. 1998b). The genetic variation in heat tolerance in grain sorghum (Sullivan and Ross 1979), soybean (Martineau et al. 1979b), various turf grass species (Wallner et al. 1982), dry beans (Schaff et al.1987), vegetables (Kuo 1992) and spring wheat (Fokar et al. 1998a) has been studied using CMS as one of the component traits. In several drought tolerance studies, CMS was assessed by inducing osmotic stress in vitro by polyethylene glycol (PEG) in crops like sorghum (Sullivan 1972), wheat (Blum and Ebercon 1981; Premachandra and Shimada 1987a), orchard grass (Premachandra and Shimada 1987b) and maize (Premachandra et al. 1989). There has been no report on the assessment of CMS under a gradual water stress condition in any crop species.

Drought stress induces membrane deterioration leading to severe metabolic dysfunction (Nir et al. 1969; Buttrose and Swift 1975). The maintenance of membrane stability during severe desiccation is important for normal physiological metabolism to continue under low water potential. Phenotype selection for CMS may not always yield accurate results for breeding purposes because of its complex nature and its strong interaction with the environment. Thus, the evaluation of this trait was done in a controlled environmental situation, and molecular markers linked to it were identified. The marker tightly linked to quantitative trait loci (QTLs) can be utilized to accumulate alleles with desirable effects through marker-assisted selection. Application of QTL analysis to study CMS will improve our understanding of genetic factors that influence this complex trait. Selection indices using marker information can be more efficient than conventional phenotype selection (Lande and Thompson 1990). Besides, mapping QTLs is the primary step for subsequent accumulation of favorable alleles by backcrossing for generating the ideal marker genotype (Stam 1994).

The recent development of high-density linkage maps in most crop species has provided the tools for dissecting the genetic basis underlying complex traits into their individual components (Lander and Botstein 1989; Tanksley 1993). The application of molecular marker techniques greatly simplifies the screening for traits that are difficult to measure (e.g. roots) and inducible in nature (OA, CMS) (O’Toole 1989; Hanson et al. 1990; Tanksley 1993). With the construction of rice molecular linkage maps the analysis of several quantitative traits under drought stress on root morphology (Champoux et al. 1995; Price and Thomas 1997; Yadav et al. 1997), root penetration ability (Ray et al. 1996; Zheng et al. 1999) and osmotic adjustment (Lilley et al. 1996) – have recently been reported. So far there is only one report on the genetic dissection of CMS by restriction fragment length polymorphism (RFLP) analysis under temperature stress in maize (Ottaviano et al. 1991). By utilizing 44 F_6 recombinant inbred lines, Ottaviano et al. (1991) detected six QTLs accounting for 39% and 53% of the phenotypic and genetic variation for CMS, respectively. There has been no report on the molecular mapping of cell-membrane stability under drought stress in any crop plants. We report here on our attempt to evaluate CMS under a natural water stress condition and to identify the molecular markers linked to genes controlling this trait in rice.

Materials and methods

Plant culture and CMS evaluation

A population of 154 doubled haploid (DH) lines was used to assess the CMS under water stress. This population was derived from culturing F_1 anthers of a cross between CT9993–5-10–1M and IR62266–42–6-2 (abbreviated as CT9993 and IR62266, respectively) at the International Rice Research Institute (IRRI), Philippines, specifically for the purpose of studying drought resistance in rice. CT9993 was originally derived from the CIAT’s (Centro International Para Agricultura Tropical) rice breeding program from a complex cross involving primarily African upland varieties (some *japonica* background in pedigree), whereas IR62266 (maternal), a lowland *indica*, was developed at IRRI (Dr. Surapong Sarkarung, personal communication). CT9993 is considered to be a drought-avoidant type because of its thick and long root system, whereas IR62266 is a drought-tolerant type due to its high OA capacity (Zhang et al. in preparation)

Seeds of 154 DH lines along with the parents were sown in the greenhouse at Texas Tech University, Lubbock, Texas, USA during the spring of 1998 in a randomized block design with three replications. At 20 days after sowing, seedlings were thinned so that each pot (30-cm diameter at the top, 26-cm diameter at the base, 31-cm height) had only three plants. Normal fertilizer practices and plant protection measures were undertaken to ensure good plant growth. The plants were watered regularly up to 50 days after sowing. During the experimental period, the average daily minimum and maximum temperatures in the greenhouse were 25°C and 35°C, respectively. The average photon flux density at noon was 1000 µmmol m^{-2} s^{-1}. Drought stress was initiated at 50 days after sowing by withholding the water supply. Leaf samples (second leaf from the top) were collected from all the plants (154×3) before the initiation of water stress. Three segments of 3 cm in length each were cut from the middle portions of the uppermost fully expanded undamaged leaf and kept in a tightly closed vial. To assess the intensity of the stress, we monitored visually plants under stress treatment and also measured relative water content (RWC) (Barrs and Weatherley 1962). The leaf samples from the stressed plants were collected at 60–65% of RWC. At this range of RWC turgor had been lost and rice plants showed wilting.

Measurements of CMS were made by following the protocol of Blum and Ebercon (1981). Samples collected were washed three times in deionized water to remove electrolytes adhered on the surface. The samples were then kept in a capped vial (20 ml) containing 10 ml of deionized water and incubated in the dark for 24 h at room temperature. The conductance was measured with a conductivity meter (YSI Model 345, Yellow Springs, Ohio). After the first measurement the vials were autoclaved for 15 min to kill the leaf tissue and release the electrolytes. After cooling, the second conductivity reading was taken. These two measurements were carried out individually for all the samples from both the control and stress treatments. The control gave a measure of leakage solely due to the cutting and incubation of leaf discs. The conductance of the stress sample was a measure of electrolyte leakage due to water stress and was assumed to be proportional to the degree of injury to the membranes. CMS was calculated as the reciprocal of cell-membrane injury after Blum and Ebercon (1981): $CMS\%=[(1-(T_1/T_2))/(1-(C_1/C_2))]\times100$, where T and C refer to the stress and control samples, respectively; the subscripts 1 and 2 refer to the initial and final conductance readings, respectively.

Data analyses and QTL mapping

All the basic statistical analyses were done using the SAS package (SAS Institute 1990). Broad-sense heritability at the genotypic mean level was computed using the formula as $h^2=\sigma^2g/(\sigma^2g+\sigma^2e/n)$, where σ^2g is genetic variance, σ^2e is error variance and n is the number of replicates.

A molecular linkage map of this population comprising of 145 RFLPs, 153 amplified fragment length polymorphisms (AFLPs) and 17 microsatellites markers was used for QTL analysis. This map was constructed at Texas Tech University to study the genetics of drought tolerance in rice (Zhang et al. in preparation). The map represents 12 chromosomes of rice and has a total distance of 1847 cM. The average distance between two markers is 5.7 cM.

QTL analysis was performed by using the composite interval mapping (CIM) (Jansen and Stam 1994; Zeng 1994). The computation was done by PLABQTL (version 1.0, Utz and Melchinger 1996), which employs interval mapping by the regression approach, as suggested by Haley and Knott (1992) using selected markers as cofactors. The CIM approach is a refinement of interval mapping where the power of QTL detection is increased by using the most significant markers as cofactors (Jansen and Stam 1994) in the regression model that allows for the reduction of possible bias in the estimation of QTL positions and effects. The threshold for QTL detection by CIM was set at a LOD score of 3.0.

The genotype data of nearest marker loci for each of nine QTLs were used for the analysis of possible digenic epistatic interaction, which was conducted by using the SAS GLM Proc (SAS Institute 1990). A probability level of 0.05 was fixed as the threshold to determine the significant epistatic effects.

Results and discussion

Relative water content and cell membrane stability

One hundred and fifty-four DH lines were grown in the greenhouse for the purpose of phenotyping CMS. Even though plants were visually monitored daily and leaf RWC was measured, not all the samples could be collected that were in the range of 60% to 65% RWC. Thus, 104 lines were screened out in this range of RWC and used for further analysis. The two parental lines, CT9993 and IR62266, were sampled at 62% and 63% of RWC, respectively. We discarded 50 lines whose RWC was recorded either high (>65%) or low (<60%). The analysis of variance showed no difference in RWC among the rest remaining 104 lines and between the two parents ($P>0.05$), which indicated that all the 104 lines along with the parents were subjected to the same level of leaf tissue dehydration.

The two parental lines differed significantly in CMS ($P<0.01$). The mean CMS values for CT9993 and IR62266 were 91.9% and 78.9%, respectively. The CMS among the 104 lines also differed significantly ($P<0.05$), and the mean values ranged from 72.0% to 96.0% with a continuous variation (Fig. 1). The continuous distribution of CMS and its broad-sense heritability (34%) indicated that CMS should be polygenic in nature. There was no significant correlation between RWC and CMS ($r=0.081$), indicating that the difference in CMS was not due to RWC. As the plants were stressed to the same level of dehydration, any difference in CMS must be due to the genetic difference among different lines. CT9993 expressed higher CMS than IR62266. Lilley and Ludlow (1996) reported that *indica* cultivars tend to be high dehydration tolerant, desiccating at a low water potential than their *japonica* counterparts. In this study, however, IR62266 desiccated first, thus it had a lower CMS than CT9993 at the same level of stress. Differences in CMS might result from differences in leaf structure (MacRae et al. 1986), cell-wall composition (Jarvis et al. 1988),

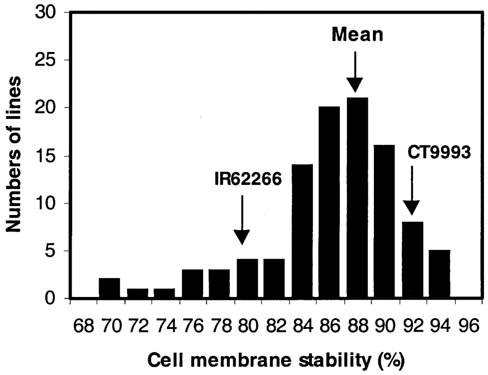

Fig. 1 Frequency distribution of cell membrane stability for 104 DH lines derived from a cross between CT9993–5-10–1-M and IR62266–42–6-2. The CMS values for two parents and the 104 lines are indicated

Table 1 QTLs detected for cell-membrane stability based on composite interval mapping (PLAB/QTL version 1.0) in 104 DH lines derived from a cross between CT9993–5-10–1-M and IR62266–42–6-2

QTLs	Marker interval[a]	Chromosome number	Distance[b]	LOD[c]	%var[d]	Add[e]
QCMS1.1	*CDO345*–ME10_14	1	5	4.97	20.1	2.35
QCMS3.1	*EM11*_2–RZ403	3	3	12.10	42.1	–2.54
QCMS7.1	*EM17_3*–ME2_15	7	6	3.18	13.4	1.74
QCMS8.1	*G2132*–R1394A	8	2	6.63	25.9	3.25
QCMS8.2	*EM18_5*–RG598	8	0	7.55	29.4	–1.75
QCMS9.1	*RZ698*–RM219	9	1	10.37	37.4	3.55
QCMS9.2	*ME9_6*–K985	9	5	3.77	15.7	–2.00
QCMS11.1	*CDO365*–ME6_7	11	2	3.42	14.3	–1.73
QCMS12.1	*EM19_5*–RG901	12	1	3.96	16.4	–2.62

[a] Marker nearest to QTL is underlined

[b] Distance from nearest marker in centiMorgans

[c] Maximum LOD scores (likelihood odd ratio)

[d] Portion of phenotypic variation explained by QTL

[e] Additive effect, a negative sign means the allele is from IR62266

Table 2 Significant digenic interactions (P<0.05) between detected QTLs as identified by SAS GLM PROC

Chromosome no.	Markers linked to QTLs	Chromosome no.	Marker linked to QTLs
1	CDO345 (*QCMS1.1*)	9	RZ698 (*QCMS9.1*)
3	EM11_2 (*QCMS3.1*)	12	EM19_5 (*QCMS12.1*)
7	EM17_3 (*QCMS7.1*)	12	EM19_5 (*QCMS12.1*)
8	EM18_5 (*QCMS8.2*)	8	G2132 (*QCMS8.1*)

the degree of membrane lipid saturation (Tal and Shannon 1983) and epicuticular wax coating (Sutter and Langhans 1982). CT9993 might be equipped with leaf features that sustain less injury than IR62266.

Mapping of QTLs for CMS

The CMS distribution is not normal (Fig. 1). Data transformation using $1/x^2$, $1/x$, $1/\log x$, $\log x$, x^2 or x^3 did not improve the distribution towards normality. Therefore, the results from the untransformed data are presented. Nine QTLs were detected for CMS, of which two each were located on chromosomes 8 and 9 and one each on chromosomes 1, 3, 7, 11 and 12 (Table 1). Both parents contributed to alleles for CMS. The phenotypic variation for CMS explained by these QTLs ranged from 13.4% to 42.1%. The LOD values for these QTLs varied from 3.2 to 12.1. QTLs *QCMS8.1* and *QCMS11.1* were located in similar genomic regions where QTLs for drought-avoidance scores existed in the CO39/Moroberekan population (Champoux et al. 1995). Five QTLs with LOD values greater than 4, i.e. *QCMS1.1*, *QCMS3.1*, *QCMS8.1*, *QCMS8.2*, *QCMS91*, each explained more than 20% of the phenotypic variation for CMS. The absolute additive genetic effects by the nine QTLs varied from 1.73 to 3.55%.

Our results show that the allelic contribution to the CMS QTLs came from both parents. A negative additive effect indicates that the source of the allele for CMS was IR62266. On the other hand, if the additive effect is positive, then the source of allele was CT9993. The allelic contribution from IR62266 to the CMS QTLs was 5 (55%) out of 9 QTLs. This was opposite to what was expected based on the parental phenotype. Even though IR62266 is phenotypically poor in CMS, it has good genetic potential for CMS. Several QTLs studies have indicated that the phenotype of a plant is not always a good predictor of its genetic potential (de Vicente and Tanksley 1993; Eshed and Zamir 1995; Xiao et al. 1997). For example, using an F_2 population of a cross between the wild tomato species *L. pennellii* and cultivated tomato *L. esculentum* de Vicente and Tanksley (1993) observed that 36% of the QTLs had alleles with effects opposite to those predicted by the parental phenotype. Similarly, in a study on rice grain yield, Xiao et al. (1997) found that alleles from the wild relative contributed to the QTLs for yield.

An analysis of digenic epistatic interactions between pairs of loci in different combinations among the nine QTLs detected the existence of four pairs of significant (P<0.05) epistatic interactions for CMS (Table 2): CDO345 (linked to *QCMS1.1*) interacted with RZ698 (linked to *QCMS9.1*); EM19_5 (linked to *QCMS12.1*) interacted with both EM11_2 (linked to *QCMS3.1*) and EM17_3 (linked to *QCMS7.1*); and G2132 (linked to *QCMS8.1*) interacted with EM18_5 (linked to *QCMS8.2*). It is difficult to interpret the real nature of these epistatic interactions in this population. As Tanksley (1993) pointed out, the development of near-isogenic lines for single and multiple QTLs will be necessary to clarify the real nature of these epistatic interactions.

Plants exhibit a number of mechanisms to maintain CMS under drought (Bewley 1979). CMS is dependent on a number of processes involving the structure of the phospholipid membrane, its arrangement with protein, its reconstitution after rehydration, lipid peroxidation, antioxidants and the production and availability of substances like sugars, polyols, amino acids and anions. An understanding of the individual genetic factors involved in all these processes will be rewarded by an improvement in CMS in the future. Maintenance of high CMS is important during a stress period for maintaining normal cellular processes. Our study found that the gene sources for CMS are from both the parents. There are several instances where alleles from phenotypically inferior parents have enhanced the trait value (Tanksley and Nelson 1996; Xiao et al., 1997; Bernacchi et al. 1998).

One of the nine QTLs found for CMS was mapped at the same locus as the OA QTL on chromosome 8 flanked by two RFLP markers (G2132-R1394 A) in this population. (Zhang et al. in preparation). Lilley and Ludlow (1996) also identified a QTL for OA in rice at a similar position on chromosome 8 flanked by two RFLP markers (RG1-RZ66). In wheat Morgan and Tan (1996) located a putative gene for osmoregulation on the short arm of chromosome 7 A. Comparative mapping indicated that the genomic region for OA in wheat and rice was homoeologous (Fig. 1 in Zhang et al. 1999). Moreover, several QTLs involved in root morphology and the drought-avoidance score in rice have been identified in this region (Champoux et al. 1995). The mapping of CMS QTLs in this region suggests that this region might contain several genes for different traits responsible for conferring drought resistance in rice. This region would be a good target for the development of drought resistance genotypes through marker-assisted selection.

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