

# QTL mapping rolling, stomatal conductance and dimension traits of excised leaves in the Bala × Azucena recombinant inbred population of rice

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## Abstract

The identification of markers linked to genes contributing to drought resistance promises opportunities to breed high yielding rice varieties for drought prone areas. Several studies using different mapping populations have previously identified quantitative trait loci (QTLs) for traits theoretically related to drought resistance. A mapping population of 176 F<sub>6</sub> recombinant inbred lines (RILs) derived from two upland rice varieties with contrasting aboveground drought avoidance traits (Bala and Azucena) with a linkage map of 157 markers was used to map QTLs for aboveground leaf morphological and physiological traits related to drought avoidance. Plants were grown for 6 weeks under controlled environmental conditions with three replications. Leaves were excised and placed on a balance. The rate of leaf rolling and water loss was recorded, after which leaf area, dry weight and specific leaf area were characterized. A simple method of estimating time to stomatal closure was employed. A total of 13 QTLs were detected for leaf morphological traits, three for initial transpiration and four for the proportion of water loss required to reach a specific advanced state of leaf rolling. No QTLs were detected for time of stomatal closure or speed of leaf rolling, nor for either water loss or transpiration at stomatal closure despite clear parental differences and moderate heritabilities in most of these traits. The co-location of QTLs for traits measured here and for drought avoidance previously reported from field experiments on chromosome 1, 3 and 5 link the genetics of drought resistance to leaf dimensions and physiology. However, a physiological explanation for a QTL for drought avoidance on chromosome 7 remains elusive.

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## 1. Introduction

Nearly half of the World's rice area relies on rainfall for its water supply and rain fed rice can experience drought at some stage of growth. A logical approach that should enable the adverse effects of drought on plant performance to be mitigated is to identify appropriate physiological mechanisms and morphological characters, then evaluate their contribution to drought resistance and yield in the target population of environments and finally use them as selection criteria in traditional breeding programmes (Cooper, 1999; Fukai and Cooper, 1995; Jackson et al., 1996; Lafitte et al., 2003; Ludlow and Muchow, 1990;

Turner, 1986). To this end, most of the traits that potentially contribute to drought resistance in rice have been reviewed (Blum, 1999; Cooper, 1999; Fischer et al., 2003; Fukai and Cooper, 1995; Lafitte et al., 2003; Nguyen et al., 1997; Price and Courtois, 1999). However, progress in improving drought resistance has been slow. This is partly due to the complexity of the drought environment, the number of different mechanisms of drought resistance exploited by rice and the interaction between the two as well as the genetic complexity of most traits. By exploiting advances in molecular biology, mapping populations have been developed and quantitative trait loci (QTLs) contributing to drought resistance-related traits are being mapped onto the rice genome (e.g. Champoux et al., 1995; Courtois et al., 2000; Price et al., 2002a).

An F<sub>2</sub> mapping population from a cross between upland indica variety Bala and the upland japonica variety Azucena was used to map QTLs for shoot traits that theoretically

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contribute to drought avoidance with particular emphasis on leaf rolling and stomatal conductance (Price et al., 1997). The F<sub>6</sub> lines derived from the same population have subsequently been used to map QTLs related to drought avoidance in field grown plants. Visible symptoms of drought avoidance, leaf rolling and leaf drying together with leaf relative water content were measured (Price et al., 2002a) as well as carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) and specific leaf area (Price et al., 2002b). In another field drought experiment leaf rolling, leaf drying, plant height and number of tillers per plant were measured (Cairns, 2003). From these data, four genomic regions in particular stand out since they repeatedly reveal QTLs for drought avoidance traits. They are near marker RZ14 on chromosome 1, near marker C136 on chromosome 3, near marker C624 on chromosome 5 and near marker C39 on chromosome 7. In only two of these regions (chromosome 1 and 5) are there evidence of (a number or notably large-effect) QTLs for root traits (Price et al., 2000, 2002c) nearby that might explain the number of drought-related QTLs detected, suggesting that the physiological mechanism behind drought avoidance at most of these regions resides in the shoot.

The parents of the Bala  $\times$  Azucena population have been shown to be contrasting for the aboveground drought avoidance-related traits. Available information suggests that Bala is more drought resistant than Azucena despite having a poor root system, since it possesses a more sensitive stomatal response to water loss, more pronounced osmotic adjustment and under drought it displays delayed leaf rolling, more rapid cessation of shoot elongation, greater response of leaf thickening and a higher yield potential (Price et al., 2002b; Laffite et al., 2004). However, the Bala  $\times$  Azucena F<sub>6</sub> population has not previously been screened for specific aboveground morphological and physiological traits that might contribute to drought avoidance. In this study the mapping population was screened in a controlled environment experiment to measure leaf traits (leaf dimensions and both leaf rolling and the rate of water loss after leaf excision) related to drought avoidance to identify genomic regions contributing to the measured traits by QTL analysis. The results are discussed in the context of previous data on drought avoidance in this population and related traits in other rice populations.

## 2. Materials and methods

### 2.1. Plant material

A subset of 176 F<sub>6</sub> randomly selected recombinant inbred lines (RILs) of the Bala  $\times$  Azucena mapping population that has previously been described (Price et al., 2000) was used in this study. The molecular map now contains 102 RFLP, 35 AFLP and 20 microsatellite markers. All 157 molecular markers are distributed among 12 chromosomes on 13 linkage groups (chromosome 12 is divided into 2 linkage groups), covering 1930 cM with an average inter-marker distance of 13.7 cM.

### 2.2. Growth conditions

Seeds from a total of 176 F<sub>6</sub> lines and parental varieties were surface sterilized in 1% sodium hypochlorite for 1 min, washed with distilled water and pre-germinated at 37 °C for 2 days. Two seeds were sown into plastic trays containing 15 pots (LBS, UK) each filled with 200 cm<sup>3</sup> John Innes No 2 potting compost. After establishment, each pot was thinned to one plant. Plants were arranged randomly in three replicate experiments, each started 1 week apart. Each experiment contained six replicates of both parents and was conducted in a growth room with controlled environmental conditions which provided 12 h daylight with 450  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR light and 70–80% relative humidity at 30/27 °C (day/night) temperatures. Three small fans provided air movement. Plants were watered daily during the first week and twice a day in the following weeks with a sprinkler. All replications received an application of 9 l of Yoshida's nutrient solution (pH 5.5) (Yoshida et al., 1976) every 4th day with a sprinkler. In addition, 9 l of Phostrogen fertilizer (A Bayer Co. Germany) (5 g in 9 l) was applied at 3 weeks. The tray positions within a replicate were rotated weekly to avoid position effects on plant growth.

### 2.3. Leaf rolling, stomatal conductance and leaf dimensions

To analyze the morphological and physiological traits 40 plants day<sup>-1</sup> were chosen randomly at the beginning of the 6th week of growth. The fans were switched off to avoid the fluctuation of readings on balances. Temperature was maintained within 1 °C of 25 °C by manual operation of the ventilation fans. One hour after the light came on, the youngest fully expanded leaves of two plants were removed from the leaf sheath using scissors and placed on each of two balances. Leaf roll score was characterized using a score of 1 (unrolled) to 5 (completely rolled) according to the method for intact plants described by O'Toole and Cruz (1980) (see Fig. 1). At the same time, leaf weight was recorded every minute for a maximum 20 min. Since 20 min were not sufficient for all plants to reach leaf roll score 5, the time taken to reach a leaf roll score 4 and 4.5 were recorded. Stomatal conductance to water vapour was measured as water (weight) loss per minute. After 20 min, leaf samples were preserved with cut ends in water for measurement of leaf area and subsequently dried to measure dry weight. A total of six parameters/traits were determined from the weight and area data. Three were calculated by developing formulae from the relationship between time in minutes and both water loss (Fig. 1) and leaf area in order to link water loss with both stomatal behaviour and leaf rolling.

Initial transpiration ( $\text{Tr}_0$ ) was determined by the equation:

$$\text{Tr}_0 = \left( \frac{\Delta W_0}{\text{LA}} \right) \times 9.26 \text{ (mmol m}^{-2} \text{ s}^{-1}) \quad (1)$$

where  $\Delta W_0$  is the rate of water loss in the first minute after leaf excision ( $\text{mg min}^{-1}$ ) and LA is leaf area ( $\text{cm}^2$ ) (a in Fig. 1).

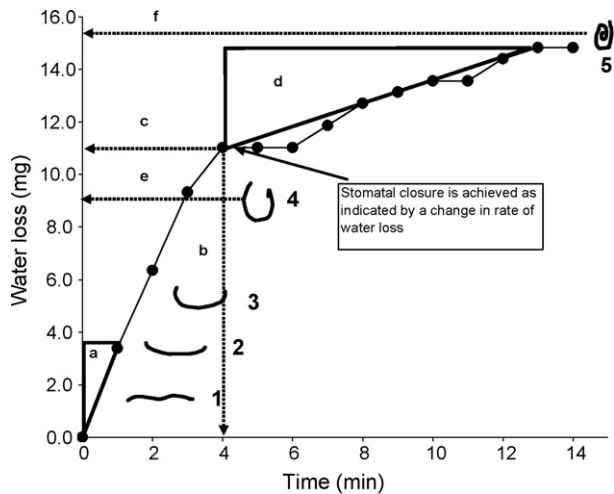


Fig. 1. Schematic diagram indicates relationship between water loss and both stomatal closure and leaf rolling: a = change in water loss during first minute after excision, b = time to stomatal closure, c = water loss at stomatal closure, d = rate of water loss at stomatal closure, e = proportional water loss at leaf roll score 4, f = water loss at leaf roll score 5. Numbers 1–5 corresponded with unrolled to rolled strands alongside the plot of water per minute represent leaf roll scores.

Time to stomatal closure (TSC) was considered as the time point at which a notable decrease in the rate of water loss per minute appeared on the plot of weight (mg) vs. time (see Fig. 1). To verify the validity of this approach, a comparison of time to stomatal closure assessed using this method and using a porometer was undertaken for 11 plants including several with high leaf rolling rates (Fig. 2). For each plant, two leaves were excised, one placed on a balance and the other clamped to the porometer (AP4, DeltaT Instruments, Cambridge). The time taken for stomata to close was obtained from the plot of either leaf weight or leaf conductance against time. We obtained a correlation of 0.923 indicating excellent agreement.

Proportional water loss at stomatal closure (PWSC) (which indicates the relationship between stomatal closure to water loss rather than time and overcomes differences associated with different rates of initial transpiration) was determined by the

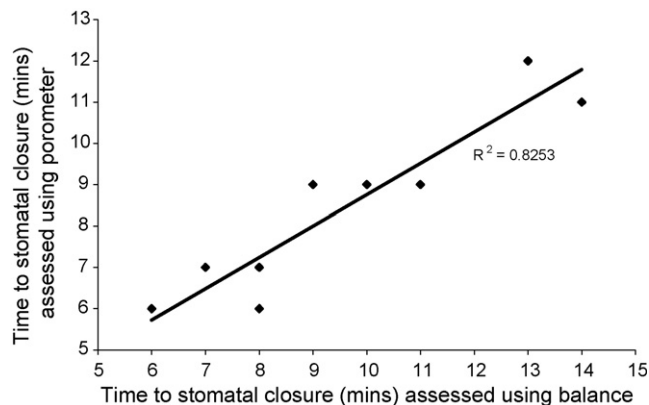


Fig. 2. Comparison of assessment of stomatal closure using a porometer or balance for 11 plants using similar-aged replicate leaves for each method.

equation:

$$\text{PWSC} = \left( \frac{\text{WSC}}{\text{TWC}} \right) \times 100(\%) \quad (2)$$

where TWC is the total water content of the leaf (the difference between initial fresh weight and dry weight of leaf; mg) and WSC is the water lost at stomatal closure (mg).

Transpiration at stomatal closure (TrSC) was determined by the equation:

$$\text{TrSC} = \left( \frac{\Delta\text{WSC}}{\text{LA}} \right) \times 9.26(\text{mmolm}^{-2}\text{s}^{-1}) \quad (3)$$

where  $\Delta\text{WSC}$  is the rate of water loss at stomatal closure ( $\text{mg min}^{-1}$ ) (d in Fig. 1). This is considered a measure of cuticular transpiration.

The PWR4 and PWR4.5 were measured as proportional water lost at leaf roll score 4 and 4.5, respectively using an equation equivalent to (2) above, but the water loss when leaf rolling of 4 or 4.5 were used.

*Note:* A conversion factor of 9.26 in transpiration calculation was determined by converting 1 mole of water (18.0 g) transpired by a unit leaf area ( $1 \text{ cm}^2$ ) per unit time (1 min) to 1 mmole of water (mmol) transpired by a unit leaf area ( $\text{m}^2$ ) per unit time (s) =  $[(1/18)/60] \times 10,000 = 9.26 \text{ mmol m}^{-2} \text{ s}^{-1}$ .

### 2.3.1. Conversion of initial transpiration into residual initial transpiration

Since a strong relationship between initial transpiration ( $\text{Tr}_0$ ) and leaf area was detected (Fig. 3), it was concluded that there was a boundary layer effect in each of three replications. Curve fitting of the relationship between  $\text{Tr}_0$  and leaf area revealed an exponential curve gave the highest  $R^2$  value (Fig. 3 presents curve for replication 3 as an example), and residual initial transpiration ( $\text{RTr}_0$ ) was calculated as the deviation from this curve. The boundary layer effect was not apparent in individual experiments when measuring water loss at stomatal closure, presumably because of the much lower rate of transpiration.

### 2.4. Statistical analysis

In order to correct for temporal variation during the period over which each replicate experiment was measured, analysis of variance and/or regression of trait values was conducted (separately) against both date and time of measurement. When the  $F$  value was greater than 10.0, indicating a moderate effect on the trait, a correction was calculated as the overall mean + (or–) the residual value from the ANOVA or regression. This affected the following traits; in replicate 1 TR4 (time), in replicate 2  $\text{RTr}_0$  (time), PWSC (time), TR4 (date and time) and PWR4 (date), PWR4.5 (date); replicate 3 PWR4 (date) and PWR4.5 (date). To calculate an average value of each RIL for each measured trait from three replications, data were normalized by dividing the value for each individual by the population mean for that trait in each replication. The average values for each individual from three replications for all traits were then calculated by averaging only those 132 RILs that

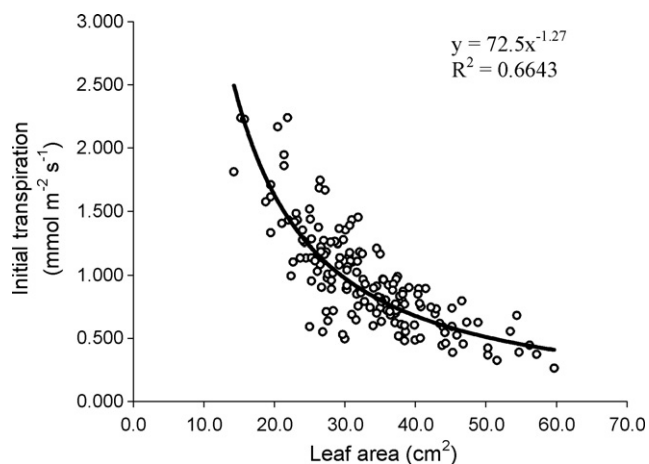


Fig. 3. Relationship between initial transpiration and leaf area for replication 3 indicating the boundary layer effect. Deviation of initial transpiration from fitted curve is used to calculate residual initial transpiration. Open circles show the plot between initial transpiration and leaf area. Bold curve is fitted exponential curve showing the relationship between initial transpiration and leaf area.

were common to all replications. The averaged data for all generations were tested for normality of distribution and transformed by square root if required. All statistical analysis was performed using MINITAB<sup>®</sup> (Release 14.12.0). The mean values of measured traits of parents and the population, and broad sense heritability ( $h^2$ ) were calculated. Broad sense heritability was computed from one-way analysis of variance (factors; genotype) of the  $F_6$  from the estimates of genetic ( $\sigma_G^2$ ) and residual ( $\sigma_e^2$ ) variance derived from the expected mean squares as  $h^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_e^2/k)$  where  $k$  was the number of replications ( $=3$ ).

One-way ANOVA was used to test differences between parental varieties. The relationship between the traits was analyzed by correlation coefficient after Pearson.

### 2.5. QTL analysis

QTL analysis was achieved by composite interval mapping conducted with QTL Cartographer version 1.15 (C.J. Basten, B.S. Weir, and Z.B. Zeng, Department of Statistics, North Carolina State University) with model 6 using the program Srmappqtl set for “forward stepwise regression with background elimination” to identify significant background markers, and having a window size of 10 cM. Permutation testing of 1000 randomizations was carried out to identify the 5% genome-wide significant threshold, which indicated a LOD score of 3.0 is suitable for this set of data.

### 2.6. Comparative mapping across populations

Comparative mapping was conducted by using two more mapping populations. The Azucena  $\times$  IR64 linkage map (Huang et al., 1994) uses RFLPs from Cornell University, whilst, the Nipponbare/Kasalath map (Lin et al., 1998) uses RFLPs from the Rice Genome Project. The integration of genetic maps was achievable since the sequence of most RFLPs

and microsatellites are now available (e.g. at the Gramene web site; [www.gramene.org](http://www.gramene.org)), sequence can be matched to sequenced BAC clones by BLAST searching at NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and the genetic location of each BAC can be found at the TIGR Rice Genome Annotation web site (<http://www.tigr.org/tdb/e2k1/osa1/index.shtml>).

## 3. Results

### 3.1. Mean values and heritability for the traits measured here

The average data for drought avoidance-related traits measured from three replications and heritabilities displayed by the  $F_6$  population for each trait are presented in Table 1. One-way ANOVA revealed that Azucena and Bala behaved differently for all traits ( $P < 0.05$  to  $P < 0.001$ ) except for residual initial transpiration ( $RT_{R_0}$ ), transpiration at stomatal closure (TrSC) and proportional water loss at leaf roll score 4.5 (PWR4.5). However, the latter was quite heritable (42.2%). Azucena had a much higher leaf area (LA) and leaf dry weight (LDW) than Bala and both traits were highly heritable (61.7% and 58.2%, respectively). Although specific leaf area (SLA) was much higher in Bala than Azucena ( $P < 0.001$ ), broad sense heritability was low (22.5%). The time to stomatal closure (TSC) was much lower in Bala than Azucena and the proportional water loss at stomatal closure (PWSC) was lower in Bala, indicating that Bala has stomata which are more sensitive to water loss than Azucena. Despite the level of statistical difference between the parents for these two traits, only PWSC displayed reasonable heritability. Azucena was significantly faster to roll its leaves to a score of 4 and lost a lower proportion of water at leaf roll score 4 (PWR4) than Bala. The broad sense heritability for time taken to reach a leaf roll score 4 (TR4) was moderate (31.0%) and for proportional water loss at leaf roll score 4.5 (TR4.5) was moderately high (51.7%).

### 3.2. Correlation analysis between traits for the $F_6$ population

Correlation coefficients for all traits are presented in Table 2. TSC was not associated with  $RT_{R_0}$  or TrSC but there was a strong correlation between TSC and PWSC ( $r = 0.527$ ,  $P \leq 0.001$ ) indicating that the plants with fast responding stomata did so because their stomata were more sensitive to water loss, not because they were losing water faster. There was, however, a significant correlation between TrSC and PWSC ( $r = 0.284$ ) suggesting that plants with a high rate of cuticular water loss had lost a lot of water before stomata finally closed. Very strong correlations between TR4 and PWR4 ( $r = 0.702$ ), and between PWR4 and PWR4.5 ( $r = 0.882$ ) were observed. The latter is not surprising since the traits are not independent, but the former indicates that leaves which rolled rapidly did so because their rolling response was more sensitive to water loss.  $RT_{R_0}$  did not correlate with the other physiological traits but did correlate weakly with LA indicating the boundary layer effect had not been completely adequately dealt with in

Table 1  
Means ( $\pm$ standard deviation) and broad sense heritability ( $h^2$ ) of aboveground drought avoidance-related traits for parental lines (Azucena and Bala) and  $F_6$  mapping population

Variety	Trait <sup>a</sup>										
	LA (cm <sup>2</sup> )	LDW (mg)	SLA (mm <sup>2</sup> mg <sup>-1</sup> )	RTr <sub>0</sub> (mmol m <sup>-2</sup> s <sup>-1</sup> )	TSC (min)	PWSC (%)	TrSC (mmol m <sup>-2</sup> s <sup>-1</sup> )	TR4 (min)	PWR4 (%)	PWR4.5 (%)	Heritability, $h^2$ (%)
Azucena ( $n = 18$ )	41.9 $\pm$ 8.6	112.8 $\pm$ 32.6	38.3 $\pm$ 1.0	-0.02 $\pm$ 0.15	7.61 $\pm$ 1.38	11.4 $\pm$ 2.0	0.22 $\pm$ 0.09	3.2 $\pm$ 1.1	5.9 $\pm$ 2.48	7.9 $\pm$ 2.3	
Bala ( $n = 18$ )	25.2 $\pm$ 6.0	56.7 $\pm$ 15.1	45.2 $\pm$ 6.0	-0.01 $\pm$ 0.51	5.61 $\pm$ 1.61	9.4 $\pm$ 2.5	0.24 $\pm$ 0.09	5.8 $\pm$ 4.1	8.2 $\pm$ 2.18	9.4 $\pm$ 2.7	
$F_6$ ( $n = 132 \times 3$ )	32.0 $\pm$ 10.0	73.6 $\pm$ 30.2	45.9 $\pm$ 9.0	0.03 $\pm$ 0.38	6.30 $\pm$ 1.62	10.5 $\pm$ 2.9	0.27 $\pm$ 0.11	4.5 $\pm$ 3.2	7.4 $\pm$ 3.14	9.4 $\pm$ 3.3	
Azucena vs. Bala	45.4 <sup>***</sup>	44.0 <sup>***</sup>	13.8 <sup>***</sup>	0.0	16.0 <sup>***</sup>	7.2 <sup>*</sup>	0.2	7.0 <sup>*</sup>	8.0 <sup>**</sup>	3.1	
Heritability, $h^2$ (%)	61.7	58.2	22.5	16.0	11.5	39.4	16.7	31.0	51.7	42.2	

Significant difference in Azucena vs. Bala are indicated by <sup>\*</sup> $F$  values from one-way analysis of variance at <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ , <sup>\*\*\*</sup> $P < 0.001$ .

<sup>a</sup> Trait are: leaf area (LA), leaf dry weight (LDW), specific leaf area (SLA), residual initial transpiration (RTr<sub>0</sub>), time to stomatal closure (TSC), proportional water loss at stomatal closure (PWSC), transpiration at stomatal closure (TrSC), time taken to reach a leaf roll score 4 (TR4), proportional water loss at time taken to reach a leaf roll score 4 (PWR4) and proportional water loss at time taken to reach a leaf roll score 4.5 (PWR4.5).

the calculation of residuals. TrSC also correlated weakly with LA and LDW indicating that, after averages were calculated, a small boundary layer effect was detectable. Weak positive correlation was detected for SLA with PWSC, PWR4 and PWR4.5 ( $r = 0.221$ ;  $P \leq 0.011$  to  $r = 0.343$ ;  $P \leq 0.001$ ) suggesting that thinner leaves required more water loss to roll. LA and LDW were highly correlated with each other with a coefficient of 0.924. The SLA was negatively associated with LA ( $r = -0.451$ ,  $P \leq 0.001$ ) and LDW ( $r = -0.693$ ,  $P \leq 0.001$ ) as expected.

### 3.3. QTL analysis

A summary of statistics of all significant QTLs (LOD > 3.0) are presented in Table 3. All these QTLs are presented graphically on the linkage map in Fig. 4, along with putative QTLs with a LOD score of 2.0–3.0 since these could be true QTLs.

#### 3.3.1. Leaf area

A total of nine QTLs were detected for LA of which four were significant and five were putative QTLs. Azucena alleles contributed for higher LA at all significant QTLs except for one on chromosome 5. Three putative QTLs, one on each of chromosomes 3 (between R1618/G164), 4 (between RG163/RM349) and 6 (near MRG6488) indicated that the Azucena parent increased LA. Two putative QTLs, one on each of chromosomes 4 (at RG190) and 6 (at R2654) were observed where Azucena alleles reduced the value of the trait.

#### 3.3.2. Leaf dry weight

A total of four QTLs were revealed with significant LOD score and Azucena was the donor for positive alleles at all loci. In addition, three putative QTLs were detected on each of the chromosome 3, 5 and 6. Of these, the Azucena allele increased leaf dry weight only for the putative QTL on chromosome 3.

#### 3.3.3. Specific leaf area

There were five regions identified with significant LOD score for specific leaf area on chromosome 1, 3, 5, 7 and 10. Three QTLs indicated that Bala alleles had a positive effect and increased specific leaf area. Out of five putative QTLs, three alleles reducing SLA came from Bala, one on chromosomes 1, 9 and 12. The remaining two putative QTLs, where Azucena alleles increased the value of trait, were detected on chromosome 2 and 4.

#### 3.3.4. Residual initial transpiration

Three significant QTLs were detected, one on chromosome 1 and two on chromosome 11. On chromosome 1 Azucena alleles increased the trait. On chromosome 11 near RM229, Azucena alleles increased RTr<sub>0</sub> and explained 11.7% phenotypic variation for the trait. However, 22 cM further down near RM206, a negative effect by Azucena alleles was detected controlling 19.4% phenotypic variation for initial stomatal conductance. Only one putative QTL was detected, where Azucena alleles on chromosome 4 increased RTr<sub>0</sub>.

Table 2  
Phenotypic correlations for the F<sub>6</sub> population between drought avoidance traits

Trait	LA	LDW	SLA	RTr <sub>0</sub>	TSC	PWSC	TrSC	TR4	PWR4
LDW	0.924***								
SLA	−0.451***	−0.693***							
RTr <sub>0</sub>	0.268**	0.158	0.088						
TSC	0.104	0.097	−0.036	0.077					
PWSC	−0.188*	−0.269**	0.263**	0.187	0.527***				
TrSC	−0.337***	−0.382***	0.156	−0.049	0.092	0.284***			
TR4	0.059	0.000	0.104	0.130	0.062	0.006	0.102		
PWR4	−0.088	−0.188*	0.221*	0.115	0.078	0.427***	0.231**	0.702***	
PWR4.5	−0.148	−0.261**	0.295***	0.074	0.099	0.486***	0.295***	0.613***	0.882***

Significant correlations are indicated \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Traits are described in Table 1.

### 3.3.5. Time to stomatal closure

Composite interval mapping detected no significant or putative QTL for TSC.

### 3.3.6. Proportional water loss at stomatal closure

Composite interval mapping detected no genomic region with significant QTL for PWSC. Two putative QTLs were detected on chromosome 7; one near C451 had positive effect from Azucena parent and other near RG650 where Azucena alleles reduced the trait. Another putative QTL was detected, on chromosome 11, and here the Azucena allele caused a reduction in PWSC.

### 3.3.7. Transpiration at stomatal closure

Composite interval mapping revealed no significant but three putative QTLs for TrSC, on chromosomes 5, 7 and 9. The TrSC was increased by Azucena alleles only on chromosome 5.

### 3.3.8. Time taken to reach a leaf roll score 4

There was no significant QTL for TR4. Of five regions containing putative QTLs, the alleles that delayed leaf rolling came from Bala in 3 of them, one on chromosome 3 and two on chromosome 5 (at markers R569 and at C624). The remaining two putative QTLs were detected on chromosomes 1 and 12 where the Azucena allele delayed leaf rolling.

Table 3  
Summary of significant QTLs for traits associated with leaf dimensions, transpiration and leaf rolling

Trait <sup>a</sup>	Chromosome number	QTL position in cM		LOD	R <sup>2</sup> (%)	Donor of positive allele <sup>b</sup>
		Above (−) or below (+) nearest marker	From the top of the linkage group			
LA	1	R117–	242	4.2	28.0	A
	2	C601 + 1	176	6.0	12.7	A
	5	RZ70 + 6	122	3.6	10.2	B
	9	G385	56	3.8	7.8	A
LDW	1	R117–13	238	6.3	27.3	A
	2	C601 + 12	188	3.5	15.3	A
	3	R1618 + 10	199	5.5	17.7	A
	11	RG2	75	3.6	6.0	A
SLA	1	a18437–1	46	4.2	8.7	B
	3	R1618–1	188	3.3	6.6	B
	5	R2232–10	20	3.9	10.6	A
	7	C39	55	4.1	6.9	A
	10	a12372–2	56	3.0	6.3	B
RTr <sub>0</sub>	1	G393	149	3.1	7.4	A
	11	RM229	75	4.5	11.7	A
	11	RM206	95	6.8	19.4	B
TSC	No QTL					
PWSC	No QTL					
TrSC	No QTL					
TR4	No QTL					
PWR4	5	C624–6	85	4.5	12.5	B
	9	G1085–4	80	3.8	9.7	A
PWR4.5	1	RG532 + 6	6	3.4	10.3	A
	2	RG520	221	3.8	8.5	B
	5	C624–5	85	5.5	15.1	B
	9	G1085–4	80	4.5	12.2	A

<sup>a</sup> Trait are described in Table 1.

<sup>b</sup> Donor of positive allele 'A' comes from Azucena and 'B' from Bala parent.

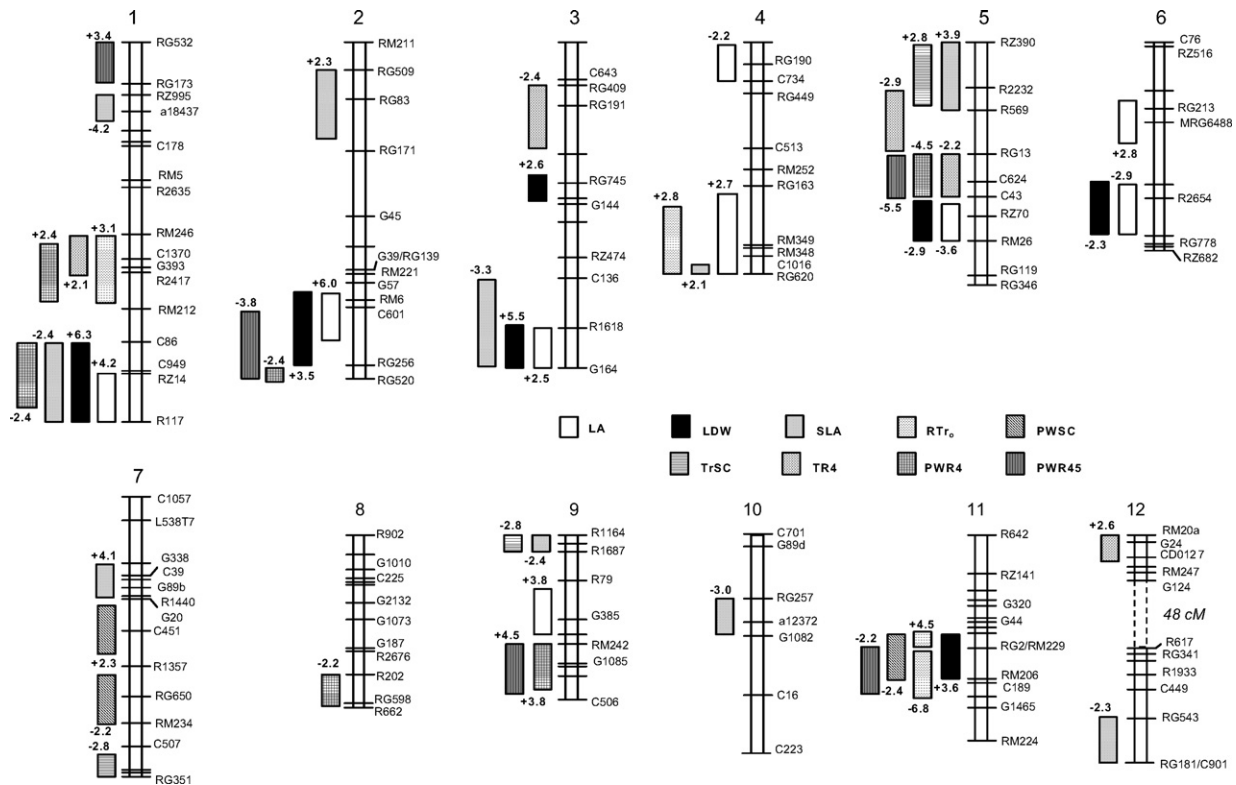


Fig. 4. Linkage map of Bala  $\times$  Azucena population (with RFLP and microsatellite and a few AFLP markers indicated) showing putative (LOD < 3.0) and significant (LOD > 3.0) QTLs for aboveground mechanisms of drought avoidance. The boxes represent  $-1$  and  $+1$  LOD interval and the number above or below the box indicates the LOD score for the QTL. A positive LOD value indicates that Azucena allele increased the value of the trait.

### 3.3.9. Proportional water loss at leaf roll score 4 and 4.5

Composite interval mapping revealed significant QTLs on identical places for both PWR4 and PWR4.5, on chromosomes 5 and 9. The positive allele for QTLs on chromosome 5 was from Bala, while that on chromosome 9 was from Azucena. Together, these QTLs accounted for about 22 and 27% of the total variation in PWR4 and PWR4.5, respectively. The region at the bottom of chromosome 2 was also detected with QTLs for PWR4 and PWR4.5 but only for PWR4.5 was it significant. The Bala allele increased the trait values at this locus. At the top of chromosome 1 there was a significant QTL for PWR 4.5 where the Azucena allele increased the amount of water lost. Of three other putative QTLs, two on chromosome 1 were for PWR4 while the one identified on chromosome 11 was for PWR4.5.

## 4. Discussion

### 4.1. Overall performance of parents Bala and Azucena

In a previous study, Price et al. (1997) mapped leaf rolling and transpiration traits on the  $F_2$  population from which the material in this study was derived. One of the main observations was that Azucena rolled its leaves faster upon excision but Bala closed its stomata faster. Despite the fact that the plants in the present study were grown under different conditions, the same conclusion is drawn. Thus Azucena leaves reach a leaf rolling score of 4 in a 3.2 min, and average of 2.6 min faster than Bala. This more rapid leaf rolling reflected a greater sensitivity of leaf

rolling to water loss, since in Azucena only 5.9% of leaf water had been lost when rolling reached 4, but in Bala 8.2% of water had been lost. In both studies, the initial transpiration rate was not different between Azucena and Bala but stomata closed more rapidly and after less water loss in Bala. Thus in this study, stomatal closure took an average of 5.6 min in Bala when 9.4% of the leaf water had been lost while in Azucena it took 2 min longer and 11.4% of leaf water was lost. These data confirm the observation that, in an excision test, leaf rolling is more sensitive to water loss in Azucena, but for stomatal closure it is Bala that is more sensitive.

In spite of these physiological differences between the parents, it may well be that plant morphology also has a contribution to drought avoidance. Parental diversity and transgressive segregation within the mapping population were observed for all morphological traits (LA, LDW and SLA) (Table 1). Bala had smaller leaves than Azucena. Bala also had thinner leaves (higher SLA), in agreement with previous data from field screening experiment, where Bala had thinner leaves than Azucena when irrigated, but thicker under drought treatment (Price et al., 2002b).

### 4.2. Mapping QTLs for aboveground drought avoidance-related traits and the evidence of pleiotropy or linkage between the traits

The  $F_2$  from which the present mapping population has been derived has previously been characterized for leaf rolling and

stomatal behaviour upon excision (Price et al., 1997). However, repeating the analysis has three major benefits. Firstly the ability to replicate increases the confidence that QTLs reported are real, secondly the molecular map is much improved and thirdly, the permanent population has a large body of additional data and QTL information that can be used to interpret results. Composite interval mapping revealed a total of 22 significant and another 30 putative QTLs for leaf area, weight, specific area (dimensions) and leaf physiology (stomatal and leaf rolling responses), many of which were not detected on screening the F<sub>2</sub> population. Twelve loci affected more than one trait. Six genomic regions were revealed where both leaf morphological and the leaf physiological traits were co-localized. This co-localization may be due to tight linkage, pleiotropy or a causal relationship between the traits. Leaf morphological traits were affected by a total of eighteen genomic regions that contained QTLs at both significant and putative LOD levels. Seventeen regions had QTLs for the leaf physiological traits with both significant and putative LOD levels.

Of five putative QTLs for time to reach leaf roll score 4, one on chromosome 1 between RM246/G393 was co-located with a putative QTL for percent water loss at leaf roll 4 and one on chromosome 5 between RG13/C43 was co-located with QTLs for percent water loss at leaf roll 4 and 4.5. The direction of the effect was consistent with the phenotypic correlation between TR4 and PWR4 suggesting that these loci delay leaf rolling by reducing the sensitivity of leaf rolling to water loss. Despite the diverse behaviour observed by the parents no significant QTL for TSC or PWSC were detected by composite interval mapping. The heritability of the former was low, either suggesting no genetic variation in the population or a flaw in the method of assessing stomatal closure. The verification experiment presented in Fig. 2 suggests good agreement between the simple weight-based method used here and that obtained with a porometer, but it may be that this method added to the environmental variation of an environmentally variable trait. Lack of QTLs for PWSC may reflect similar shortcomings of the method of assessing stomatal closure, yet heritability was not negligible suggesting lack of QTLs reflects the action of only small-effect loci.

#### 4.3. Noteworthy genomic regions

Of twelve identified regions in the present study, which influenced more than one trait, three are considered noteworthy since they confirm the relationship between morphological or physiological traits of excised leaves measured here and previously mapped drought avoidance-related traits on the mapping population. Below we focus on these in more detail because, by doing so, a better insight into potential physiological processes affecting drought avoidance in the field should be obtained. This improved functional understanding may also inform the hunt for candidate genes underlying QTLs, since the gene function is more directly assessed. Here we consider QTLs are co-localized if they have overlapping one-LOD intervals.

##### 4.3.1. Chromosome 1

The genomic region between C86/R117 at the bottom of chromosome 1 was revealed with large-effect QTLs for several traits. The Azucena allele increased leaf area and leaf dry weight and perhaps reduced specific leaf area and proportional water loss at leaf roll score 4 measured here. In the Bala × Azucena mapping population QTLs for drought avoidance traits in the field have been detected here, where the Azucena allele; reduced leaf rolling and drying, while increasing relative water content (Price et al., 2002a); increased plant height, panicle length and grain weight, biomass and spikelet sterility under stress while reducing harvest index (Laffite et al., 2004). In this population this is a QTL for plant height with the Azucena allele being positive (MacMillan et al., 2006). A number of QTLs for root morphology have been detected here and the Azucena allele increased the root growth under well-watered conditions and reduced the traits under drought (Price et al., 2002c). A similar concentration of drought and root-related QTLs has been observed in this region in the well studied Azucena × IR64 population (e.g. Courtois et al., 2000; Yadav et al., 1997). The deletion mutant of the semi dwarf gene *sd1* that has been cloned (Sasaki et al., 2002) is present in Bala (data not shown) and maps 3 cM above RZ14 in this map and this may be responsible for the large amount of QTLs related to drought avoidance and leaf dimension traits observed in this region. However, Ishimaru et al. (2001a,b) have detected QTLs for plant height and adaxial stomatal (respectively) in a similar region of the Nipponbare/Kasalath population. Subsequently Ishimaru et al. (2004) demonstrated that the gene underlying the height QTL is sucrose phosphate synthase which is 10 cM lower than *sd1* in the genome sequence. This suggests the real possibility that the concentration of QTLs for shoot morphological and physiological traits at the bottom of chromosome 1 in rice may be due to more than one gene. This will require meta-analysis like that described by Goffinet and Gerber (2000) to elucidate.

##### 4.3.2. Chromosome 3

A number of leaf dimension traits were associated with a region at the bottom of chromosome 3 between C136/G164. The Azucena allele increased leaf area (putative), leaf dry weight and reduced specific leaf area measured here. In previous field screens on this population the Azucena allele at this QTL increased carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) (Price et al., 2002b), leaf rolling and leaf drying in the field droughted plants (Price et al., 2002a), grains per panicle and panicle length under water stress and biomass under well-watered conditions (Laffite et al., 2004). However, the Bala allele increased the values for relative water content (Price et al., 2002a), individual grain weight, flowering delay, panicle number (Laffite et al., 2004) and tiller number (Cairns, 2003). This QTL has also been mapped in other mapping populations for several traits. This genomic region in the Azucena × IR64 population revealed QTLs for leaf rolling (Courtois et al., 2000), plant height, tiller number (Hemamalini et al., 2000), number of panicles, panicle length and total grain weight (Hittalmani, et al., 2003). This locus in the population from Nipponbare/Kasalath was detected



for plant height, Rubisco to chlorophyll ratio and carbon isotope discrimination (Ishimaru et al., 2001a). We would conclude that this locus clearly affects plant behaviour under drought, but the precise response appears to differ under different genetic and environmental conditions.

#### 4.3.3. Chromosome 5

The genomic region on chromosome 5 between RG13/C43 appears to have two distinct QTLs in this study, the upper affecting leaf rolling in excised leaves, and the lower the leaf dimensions. This may help elucidate some contradictions observed in drought avoidance QTLs detected in this region. For example, in Price et al. (2002a), QTLs for relative water content of droughted plants were observed between C624 and C43, (the Azucena allele increasing water status) but both positive and negative effect QTLs for leaf rolling were detected. At one field site Azucena alleles at C624 increased leaf rolling but at the other site, C43 decreased leaf rolling. It was speculated that there may be two separate loci here rather than a locus by environment interaction. A plant height QTL is detected here that coincides with a QTL affecting root morphology, with the Bala allele increasing height and root length (e.g. MacMillan et al., 2006). While meta-analysis will be required to be confident, evidence suggests there is an upper locus affecting leaf rolling and water loss, and a lower one affecting plant height, leaf dimensions and root traits, indicating no pleiotropic link between these traits in this region.

#### 4.3.4. Chromosome 7

The region between G338/G20 on chromosome 7 is interesting due to evidence that this locus has an effect on drought avoidance across environments and populations that is independent of root morphology or plant height. The Azucena allele increased leaf rolling and reduced leaf drying in field droughted plants (Price et al., 2002a); the opposing direction of effects for both the traits in the same environment implies a negative physiological relationship between them. This QTL was also detected for leaf rolling in the Azucena × IR64 population (Courtois et al., 2000; Hemamalini et al., 2000). In both the populations, the Azucena allele increases leaf rolling suggesting this as an important region in drought avoidance. In further drought screening experiments in the field, Cairns (2003) reported this QTL where the Azucena allele appeared to increase drought avoidance by reducing both leaf drying and leaf rolling. The conflict in the direction of effects between leaf rolling and leaf drying in the same experiment, or for leaf rolling between experiments is hard to explain. While the Azucena allele has been shown to increase biomass in the Bala × Azucena population in relatively stressed conditions (Laffite et al., 2004), it decreased it in well-watered conditions in an experiment with the IR64 × Azucena population (Hittalmani et al., 2003) adding to the difficulty is assessing the underlying physiology at work here. This locus was not detected for any physiological trait of drought avoidance measured in the present study, but one QTL for specific leaf area was revealed where the Azucena allele produced thinner leaves so little new insight has been advanced for this locus. In

order to dissect this QTL, recombinant inbred near isogenic lines (RINILs) have been developed by marker-assisted selection. Physiological experiments with these RINILs are underway.

## 5. Conclusion

We have observed divergent leaf rolling and stomatal behaviour of excised leaves in relation to water loss for two drought resistant rice varieties Azucena and Bala, in good agreement with a previous study. QTL results from composite interval mapping analysis of these physiological phenomena and leaf morphological traits enabled us to begin to link morphological and physiological traits with previously mapped drought avoidance QTLs in the Azucena × Bala population. The notable association of some drought avoidance QTLs with QTLs for leaf dimensions suggest that they have a causal relationship. For the QTL for drought avoidance on chromosome 7, no new insight into the underlying physiology has been gained. This study has, however, increased our confidence in suggesting that genomic regions on chromosomes 1, 3 and 5 affect drought avoidance via alterations in leaf dimensions and leaf rolling behaviour and are good targets for marker-assisted selection aimed at improving drought resistance. This would help to improve rice varieties for drought prone areas.

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