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GENOME-WIDE ASSOCIATION MAPPING REVEALS A RICH GENETIC ARCHITECTURE OF DROUGHT RESISTANCE TRAITS IN RICE (*Oryza sativa* L.)

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ABSTRACT

Improving the drought resistance of high yielding rice (*Oryza sativa* L.) varieties for areas prone to drought is a goal of rice breeders. Identify the location of genes that impart to drought avoidance in a quantitative manner should capable the using of these genes in plant breeding and speed up this goal. A total of 328 cultivars of the Diversity Panel Rice have been assessed for drought avoidance. Quantitative trait loci (QTLs) for the visual scores of leaf rolling were identified by using efficient mixed model analysis (EMMA). QTLs were considered reportable if they had *P* values (below 0.0001).

The most significant SNP associations (EMMA4.2, EMMA6.2 and EMMA7.1 respectively) were in each of the rice chromosomes 4, 6 and 7. All genes positioned 200 kb around associations were selected. The candidacies of the most promising were Zinc finger proteins (Os04g53700), organic cation transporter protein (Os04g53930) and bZIP transcription factor (Os06g41770) which has been expressed in leaf tissue and need to be investigated further.

INTRODUCTION

Drought is the most crucial factor limiting plant production. The deficiency of water worldwide and uneven distribution of rainfall makes the improvement of drought tolerance particularly important (19). Price and Courtois (1999) reported that within a physiological linguistic context, the mechanisms of drought resistance can be classified into four: drought escape, drought avoidance, drought tolerance and drought recovery. Plants have several defense mechanisms against unfavorable environmental circumstances such as increasing root depth (26), stomatal closure (32) and leaf rolling (12, 24).

Leaf rolling is the first visual symptom of drought seen in rice (3). It plays an important role in drought resistance in numerous species in the Gramineae. During stress it decreases the leaf surface area exposed to sun light and decreases transpiration so that gaseous exchange is decreased and photosynthesis is reduced. The main hindrance in drought resistance breeding is the poor understanding of the genetics and inheritance of drought tolerance traits. Efficient screening techniques are a pre-requisite for success in selecting desirable genotypes through any breeding programme (21). Different breeding approaches for drought resistance have emerged. However, most breeding attempts remain centred on enhancing productivity under favourable environments where genetic variance, heritability and therefore breeding progress for grain yield are greatest (28). Experiments under field drought conditions are often the only systematic method exploited to raise yield stability of new crop varieties in drought prone environments (29).

There are a number of studies on the variability of various components of rice physiology that contribute to drought resistance. A study by Abd Allah (1) using F_2 and F_3 lines derived from crossing made between three drought

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resistant (IET 1444, Moroberekan and Gaori) and two susceptible (Sakha 101 and Sakha 102) parents, reported that plants which showed more leaf rolling appeared to be less drought resistant. The mapping of genes that contribute to drought avoidance in a quantitative way should provide a strong basis for the exploitation of these genes in breeding through marker-assisted selection, and may result in uncovering gene identity and function (25). Several studies on QTL mapping of drought avoidance traits have been conducted in rice. Champoux *et al*. (4) conducted a field experiment in the Philippines on the CO39 × Moroberekan mapping population and revealed 18 regions contributing to leaf rolling. The Azucena x IR64 population has been evaluated for leaf rolling, leaf drying, relative water content of leaves and relative growth rate under water stress revealing 11, 10, 11 and 10 QTLs respectively for the traits (6). Gomez *et al*. (4) detected QTLs for leaf rolling on chromosomes 6 and 11 and two QTLs on chromosome 2 near markers RM6836, L14-475 and RM263 and RM240 respectively by using a total of 250 recombinant inbred lines of F_8 plants generated from crosses between two *Indica* rice lines IR20 and Nootripathu.

The Bala x Azucena population has also been extensively studied. QTLs associated with leaf rolling in excised leaves have been identified in rice (26). The same population has been tested for drought avoidance in two different places in the Philippines and West Africa with two dry seasons, revealing 17 QTLs for leaf rolling, leaf drying and relative water content (25). Cairns *et al*. (2009) reported that a mapping population of 114 lines from Bala and Azucena was grown under drought stress at two field sites with contrasting soil physical properties, drought was applied between 35 and 65 days after sowing and leaf rolling and drying were assessed. The population has also been assessed in field drought conditions in Tamil Nadu (10) and in the Philippines tested a six progenies of cross between Bala and Azucena and they found that mapping populations can provide relationships between yield components and secondary traits (16). A meta-analysis of the drought-related QTLs in the Bala x Azucena population was provided by Khowaja *et al*. (14).

A study by Zhou *et al*. (42) suggested that the exploration of new genes controlling rice leaf rolling is an important basis for rice functional genomics and plant architecture improvement. They identified a rolling leaf mutant from the *Indica* variety Yuefeng B, named *rl11* (*t*), and fine mapped the mutation between markers RM6089 and RM124 on chromosome 4. The locus seemed to reduce plant height, leaf rolling and leaf width.

In the present study, a rice diversity panel was exposed to drought and leaf rolling was scored to identify QTLs and candidate genes related to drought resistance. There are no reports of QTL mapping using GWAS in rice and it is anticipated that this study will provide both a high number of QTLs given that many alleles are present in the population, and that the accuracy of identifying QTL location will greatly aid the identification of candidate genes.

MATERIALS AND METHODS

Plant material and set up of the experiment

A total of 371 cultivars of the Rice Diversity Panel (http://www. ricediversity.org/) were received from the Susan McCouch, Cornell University, USA and multiplied in Aberdeen University in the summer of 2008. A subset of 328 accessions was used for this experiment. Appendix 1 presents the population and the subspecies to which they belong. Of those tested, 277 belonged to the rice subpopulations *aromatic* (9), *aus* (53), *indica* (57), *temperate japonica* (75) or

tropical japonica **(85). The remaining 48 were classed as admixtures between subpopulations (Figure 1). The layout of this experiment was a randomized complete block design with four replications. The design of blocks was arranged linearly along the length of the box in the North-South orientation that was employed. One soil-filled box with 450 cm length, 90 cm width and 40 cm in depth was prepared. A total of 328 rice diversity panel accessions were sown on 26th August 2011.Supplementary light of 150 µmol m^{-2} s^{-1} PAR was supplied for 12 hours a day within temperature range from 28 - 30°C. At 56 days after sowing the water was withheld for 25 days. Leaf rolling score was recorded. Theta probes reading were taken regularly.**

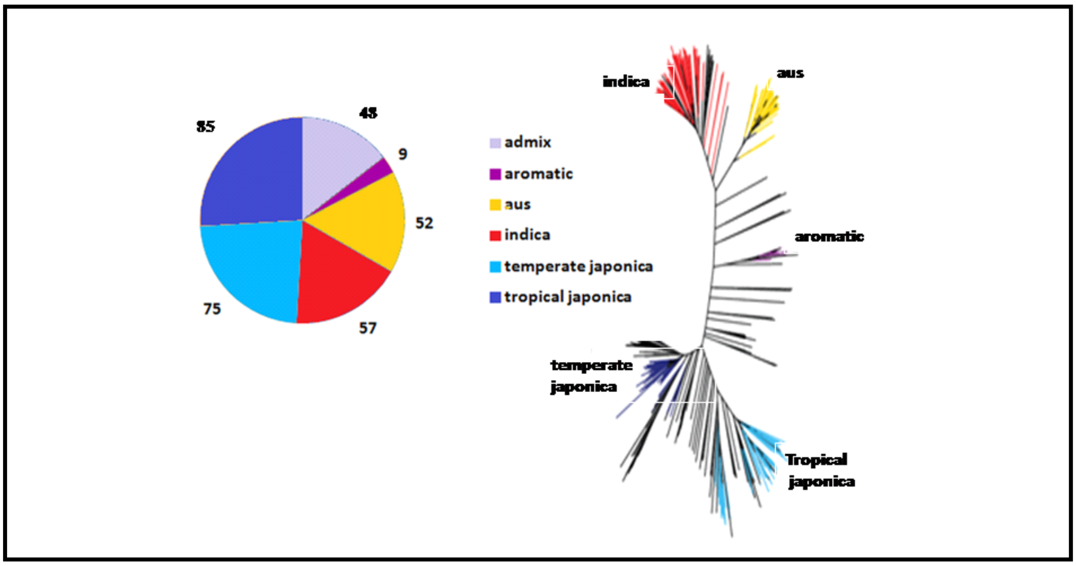

Figure 1: The left pie chart displays the distribution of subpopulations in the 327 *O. sativa* samples used in this experiment. The size of the pie chart is proportional to the sample size and colours within each pie chart are demonstrative of the proportion of samples in each subpopulation. The right phylogenetic tree and the branch tips of the tree are coloured according to the subpopulation which are *indica*, *aus*, *aromatic*, *tropical japonica* and *temperate japonica* (Source: Zhao *et al*., 2010).

Association mapping in rice

The rice diversity panel have been genotyped at Cornell University, New York on an Affymetrix genotyping array which comprises of 44,100 SNPs distributed over the rice genome (380 Mb) (41). Tung *et al.* (34) reported that with ~1 SNP/10 kb coverage was estimated for this SNP chip. All the analysis for association mapping was performed by Dr. Alexander Douglas Statistician and Bioinformatician (University of Aberdeen) by using statistical package R. An efficient mixed model analysis (EMMA) taking population structure into account was done on all the genotypes following the methodology reported by Kang *et al*. (13), which was modified from Yu *et al*. (39) who developed a novel mixed-model approach to simultaneously account for multiple levels of relatedness detected by random genetic markers. Based on data from a maize association mapping project, this approach has excellent type I and type II error rates. In addition, this technique should be readily applicable to a wide range of species and populations, as it estimates population structure based on increasingly available molecular marker data.

Separate analysis without population structure was conducted on each of the four most numerous subpopulations separately. This analysis with EMMA plus separate sub-populations is identical to the statistical approach adopted by Zhao *et al*. (41) in the first publication mapping traits using this SNP data on this population. For the result, Dr Douglas provided several files including four pdf files (Histogram, Manhattan mixed, Manhattan naïve and QQ plot). In the present study the mixed models file (text file) that resulted from EMMA was first examined which included a *P* value, SNP identification, bp position (on the rice genome in base pairs) and chromosomes name for each SNP. Also we used the statistic for the minor allele frequency value which was obtained from previous analysis of other data, and relates to the allele of the SNP that is of lowest frequency within the genotypes. If this proportion was less than 5%, it will mean that SNP is potentially not reliable. For the analysis of the separate subspecies, the aromatic and admixtures were removed from the data set prior to association mapping analyses as there were inadequate numbers of individuals within these groups. The four groups of association populations were analysed for association mapping. Zhao *et al*., (41) suggested that there was no uniform threshold *P* value that can be considered in genome wide association mapping. Here, QTLs were considered reportable if they had multiple close (within 200 kb) SNPs with low *P* values (below 0.0001) and where at least some of these SNPs did not have minor allele frequencies below 5%.

Candidate gene compilation

Based on the approach of Zhao *et al*. (41), genes situated approximately 200 kb around associations (excluding transposons) were considered positional candidates (assuming LD of 200 kb). Therefore, lists of genes within this region were collected using the rice Pseudomolecule version 6 from the Rice Genome Annotation Project. In order to gather more information about candidate genes, the expression pattern of each was assessed bioinformatically using the rice expression profile database (RiceXPro) http://ricexpro.dna.affrc.go.jp/ after converting Rice Genome Annotation Project (RGAP) names to International Rice Genome Sequencing Project (IRGSP) names at http://rapdb.dna.affrc.go.jp/tools/converter/run. In addition, the candidate genes with clear expression in roots were investigated further in the literature to determine whether they are related to cell expansion or leaf tissue in other studies which would make them particulary good candidate genes.

Leaf rolling score

A leaf rolling score was taken at 25 days of water stress. The visual score of the degree of leaf rolling or folding was made on the plants using a scale from 1 to 5 (O'Toole and Cruz, 1980), where score 1 indicates no symptoms, 2 was the first evidence of rolling, 3 was half leaf rolled, 4 was the whole leaf was folded (e.g., the two sides come together) and 5 being a tightly closed cylinder (Figure 2).

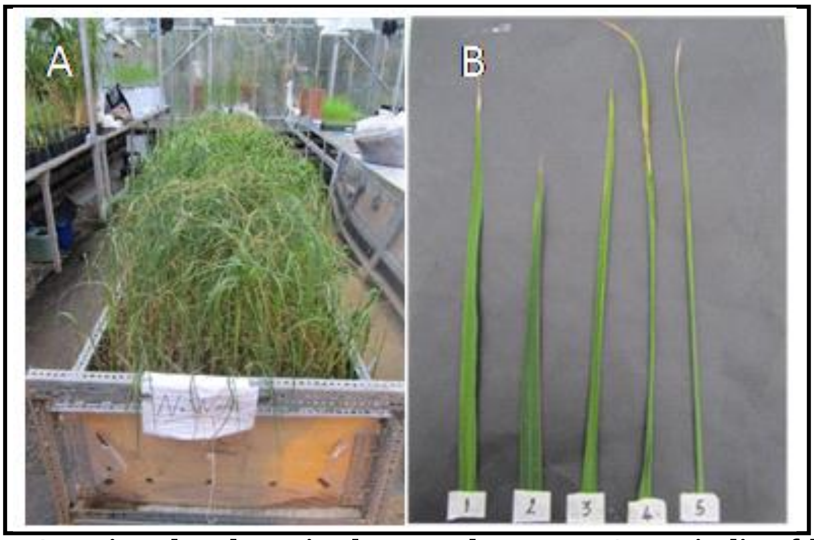

Figure 2: A; Growing the plants in the greenhouse; B; Score indix of leaf rolling from 1 to 5 where score 1 was no symptoms, 2 the first evidence of rolling, 3 when half the leaf rolled, 4 was the whole leaf folded and 5 being a closed cylinder.

Statistical and bioinformatic analysis

Minitab version 15 was use to analyse the data. Two-way ANOVA with factors genotype and block was utilized. The data were corrected for the block effect and also for normality by using base $\log_{10}$. The significance of differences between the cultivars in the leaf rolling score was tested using one way ANOVA. The association mapping analysis was done using a mixed model analysis (EMMA). Trait-marker associations were considered reliable with *P* values below 0.0001. The significant SNPs were tested for minor allele frequencies, with values above 5% considered to be dependable. Candidate genes were selected as positional candidates when they were located with 200 kb of the QTL identified.All the candidate genes have been tested for full-length cDNA (fl cDNA) and expressed sequence tag (EST) (2).

RESULTS AND DISCUSSION

Score of leaf rolling

The score of leaf rolling was recorded at 25 days after water stress. One way ANOVA showed the differences in leaf rolling between the cultivars to be highly significant ($P = 0.001$, $F = 2.24$, $R^2 = 25.29$). The rice cultivars Tsipala421, Maratelli, Tamaotsao, CS-M3, Bombon, Wells, Guineandao, Victoria F.A and Azucena had the highest score for leaf rolling while Bala, Irga 409, Saraya, WC 4443, Zhe 733, Zhenshan 2 and Kotobuki mochi the lowest Alshugeairy, 2013). Figure 3 demonstrated the frequency of score for leaf rolling, cultivars explained 25.3% of the variation within the population. Figure 4 showed that there was significant variation between rice subpopulations in leaf rolling score ($p<0.001$, $F = 17.22$, $R^2 = 24.5\%$). The graph revealed that *admix* and *tropical japonica* (TRJ) groups had the highest means (2.27 and 2.10 respectively) while the *aus* and *indica* had the lowest mean (1.59 and 1.51 respectively) and the mean score of leaf rolling for *aromatic* and *temperate japonica* (TEJ) were 2.08 and 2.00 respectively.

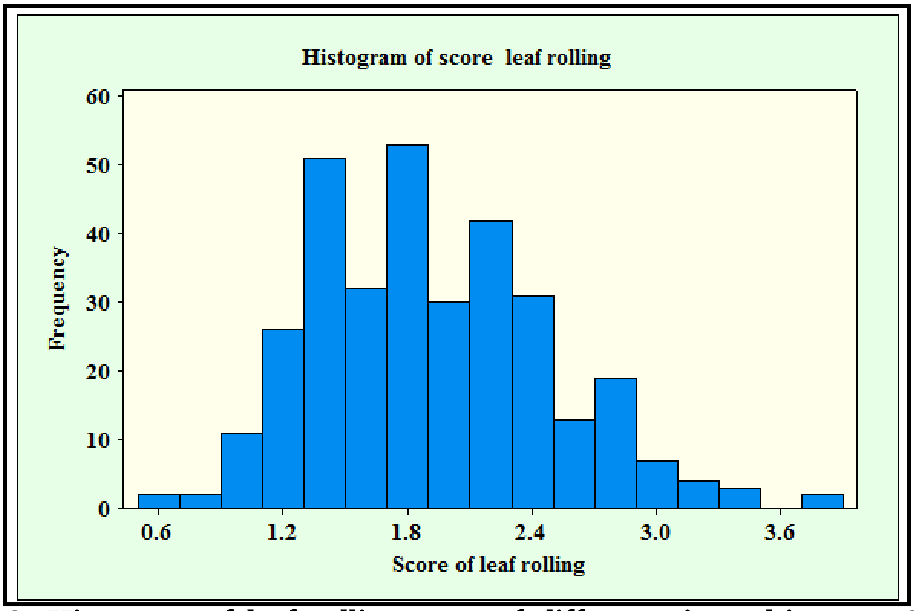

Figure 3: Histogram of leaf rolling score of different rice cultivars at 25 days after water stress.

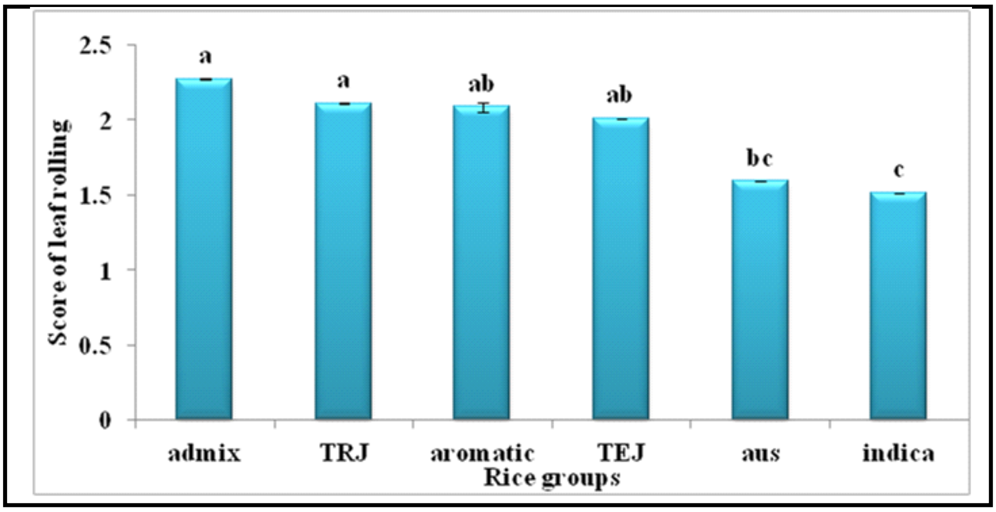

Figure 4: Mean score of leaf rolling for rice subpopulations [*aromatic* (n= 9), *aus* (n = 53), *indica* (n = 57), *temperate japonica* (TEJ) (n = 75), *tropical japonica* (TRJ) (n = 85) and *admix* (n=48)] with standard error bars. The bars that do not share a letter are significantly different as assessed using Tukey's test.

Associations of leaf rolling

Cumulative distributions of *P* values in a genome-wide scan for leaf rolling score at 25 days of water stress are shown in Figure 5 which shows that the EMMA analysis is efficient in removing large numbers of false positive tests observed in the naive analysis. Figure 6 shows the results of the GWAS analysis of the rice diversity panel using EMMA. A total of three SNPs were detected with a *P* value of < 0.0001 (- Log_{10} *P* = 4) which look like QTLs (there are multiple SNPs arising and going down in graphs) (Figure 7, Table 1). The three most significant SNP associations (EMMA4.2, EMMA6.2 and EMMA7.1 with minor allele frequnct 0.29, 0.27 and 0.21 respectively) were in each of the rice chromosomes 4, 6 and 7. For each of these locations the genes within 200 kbp are listed (2). A total of 27, 31, 23 and 19 respectively, significant SNPs associated with score of leaf rolling were detected from data of association analysis for

individual subpopulations *indica*, *aus*, *temperate japonica* and *tropical japonica* (Figure 6). In current study only those SNPs detected in the mixed model have been taken forward for listing candidate genes.

It is noteworthy that Khowaja *et al*. (14) detected QTLs related to drought avoidance on chromosomes 4, 6 and 7 near markers RM349, RZ682 and RM234, respectively with physical positions 32499412 - 32499619, 26707816 - 26707970 and 25472688 - 25472820 respectively. These are close to the physical position of EMMA 4.2 at 31746611 bp, EMMA 6.2 at 24934849 bp and EMMA 7.1 at 24597638 bp and may therefore represent the same QTLs.

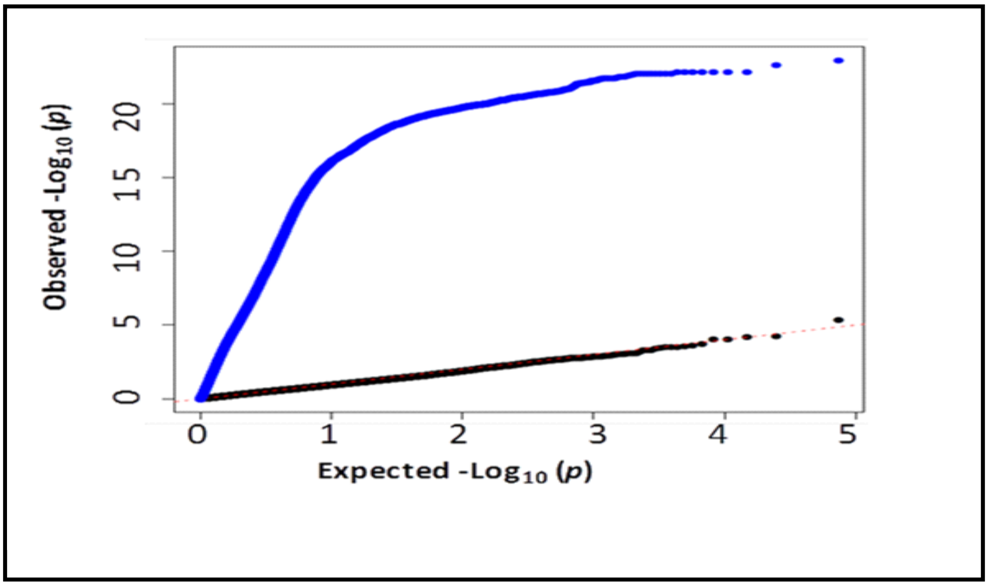

Figure 5: Cumulative distributions of *P*-values in a genome-wide scan for leaf rolling score at 25 days of water stress. Blue dotted line is the naïve data plot, Black dotted line is the data corrected for population structure using efficient mixed-model association (EMMA). The red dotted line is a y = x plot.

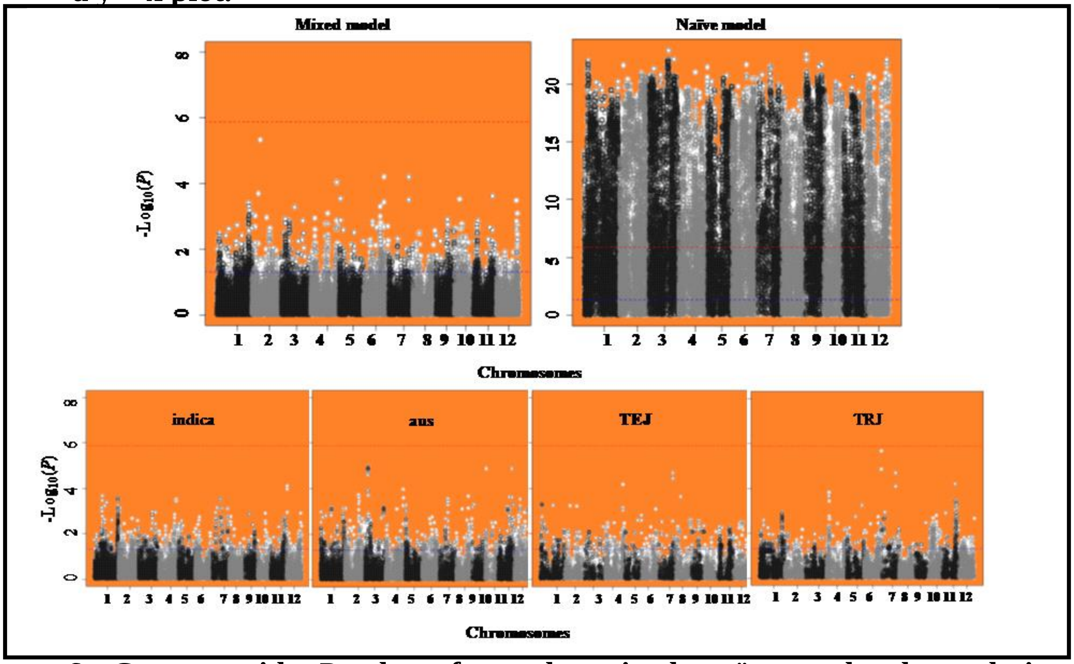

Figure 6: Genome-wide *P*-values from the mixed, naïve, and subpopulation methods. The *x* axis shows the SNPs along each chromosome; *y* axis is the $-Log_{10}$ (*P*-value) for the association.

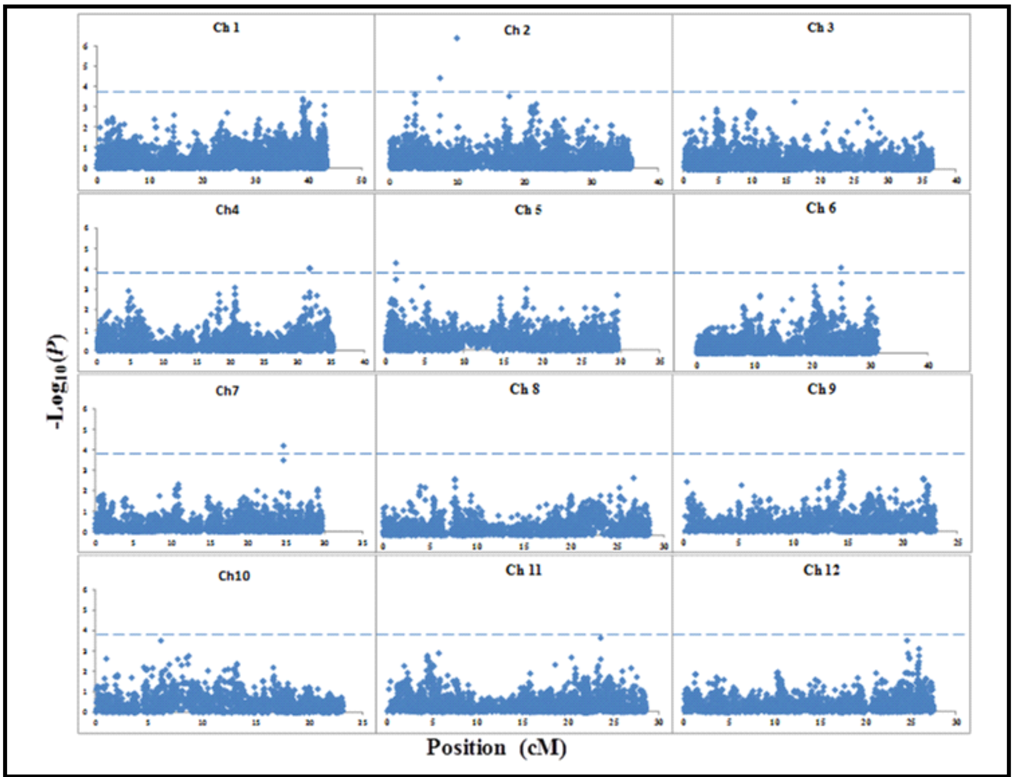

Figure 7: Genome-wide associations of leaf rolling score. Data corrected for population structure by efficient mixed-model association. Dashed line shows threshold at - $\log_{10}$ (*P*-value) = 4.

Table 1: The highly significant SNPs with genome-wide associations' for leaf rolling score.

log	P value	Position (bp)	SNP id	Chromosomes	Association name
4.04	9.1E-05	31746611	id4010930	4	EMMA4.2
4.19	6.5E-05	24934849	id6013323	6	EMMA6.2
4.20	6.3E-05	24597638	id7004524	7	EMMA7.1

By assessing leaf rolling in 328 accessions it was possible to show that there was significant variation in the population. The differences observed in leaf rolling between the rice varieties reflects either difference in leaf water content or in responsiveness to water loss. Dingkuhn *et al*. (7) reported differences in score of leaf rolling between semidwarf (IR20) and Azucena cultivars of rice. Singh and Mackill (33) reported that delayed leaf rolling (rolling at low soil water potential) was positively related to drought resistance. They reported that leaf rolling was positively related to soil water potential in the top 30 cm of soil, not to leaf water potential.

The results indicate noticeable subpopulation differences in leaf rolling structure in these accessions of rice (Figure 4). The *indica* subpopulation has significantly lower leaf rolling score compared to other rice groups except *aus*. This may be due to the higher stomatal density and greater resistance to high temperature in *Indica* compared to *Japonica* rice subspecies as reported by Maruyama and Tajima (20). Many rice cultivars belonging to *Japonica* subspecies have been tested by Lin (17) for leaf water potential and leaf rolling who found that under drought conditions, *Indica* cultivars exhibit lower leaf water potential and a lower degree of leaf rolling than those of *Japonica*. He

suggested that the sensitivity of leaf rolling to drought stress in *Japonicas* is more than *Indicas* and the ability to recover from stress is higher in *Indicas* than that in *Japonicas* which matches the results obtained here.

The candidate gene lists for the three SNPs associations revealed using the mixed model analysis approach (2). All genes positioned 200 kb around associations were selected. A total of 20 genes from the candidate gene lists are annotated as 'hypothetical proteins', and 3 as 'conserved hypothetical proteins'. The RiceXPro database shows that many candidate genes are expressed in leaf tissue (Alshugeairy, 2013). Of these, a total of 95 candidate genes were expressed in leaf tissue and other plant tissue, 49 did not show any expression and 11 are expressed in other plant tissue, but not in leaves. Those that are expressed in leaf tissue are particularly good candidates and these were investigated further in the literature. Zinc finger proteins (Os04g53700) are considered as one the major families of transcription factor regulatory proteins, responsible for the regulation of several elementary processes of plant growth (5). The database RiceXPro showed that this gene had expression intensity in leaf tissue reaching roughly 300 Cy3. Investigations on the *IFL1* (*Interfascicular Fiberless 1*, also called *Revoluta*) genes of Arabidopsis or rolled leaf (*RLD1* and *RLD2*) genes in maize have shown that the regulatory role in vascular differentiation has been proven to be played by members of the small class III homeodomain leucine-zipper (HD-ZIP) proteins that belong to the zinc-finger family (43).

Another candidate gene from the EMMA analysis is organic cation transporter protein (Os04g53930). RiceXPro suggested this gene had expression intensity in leaf tissue that reach of about 10,000 Cy3. In *Arabidopsis* there are a total of six genes in the gene family of organic cation transporters (OCTs) (15). However in plants little is known about this gene family in respect of function, localisation and regulation. Only one *OCT* has been characterized as a carnitine transporter at the plasma membrane, this was shown to have a role in stress adaptation, as its expression is up-regulated after drought stress. Controlled transport processes via membranes of different compartments and among organs are essential for plant nutrient and ion distribution. For such transport process several substrate specific membrane proteins have been detected at the tonoplast. These are instances for transporters for water and organic solutes, like urea, sugars and sugar alcohols (15). Sugars and particularly glucose are the preferred organic solutes for osmotic adjustment in cells, allowing the plant to continue water uptake under drought stress (21).

bZIP transcription factor (Os06g41770), is a member of the basic leucine zipper (bZIP) transcription factor family. A total of 74, 89 and 88 bZIP genes were identified in *Arabidopsis*, rice and poplar respectively by Ji *et al*. (11). In addition, a total of 26 rice bZIP transcription factor genes, including OsbZIP16, have been revealed to be up-regulated at least 2-fold under drought stress (22). Expression of OsbZIP23 is strongly regulated by a wide spectrum of stresses, including drought, salt, ABA, and polyethylene glycol treatments, whilst other stress-responsive genes in the bZIP family are somewhat induced only by one or two of the stresses (36). RiceXPro showed that this gene had experssion intensity in leaf tissue of roughly 2000 Cy3.

Several studies have identified QTLs associated with leaf rolling in rice. Xu *et al*. (37) detected QTLs for leaf rolling on chromosomes 1, 3, 4, 5, 6, and 7 in the Lemont x Teqin population cultivars. Courtois *et al*. (2000) detected 11 QTLs for leaf rolling in rice, two of these on chromosome 4 and 7, by testing of 135

doubled haploid lines derived from crossing between IR64 and Azucena cultivars. There are another two rolling leaf genes, *rl7* and *rl8,* on chromosome 5 (31). Moreover, fine mapping of a further leaf rolling gene, namely *rl* $_{(t)}$, on the long arm of chromosome 2 was reported by Shao *et al*. (30). Yan *et al*. (38) identified a rolling leaf mutant *rl9* (*t*) from Zhonghua 11 on chromosome 9. Luo *et al*. (19) defined a rolled leaf gene *Rl10* (*t*) on chromosome 9. Yu *et al*. (40) identified a unilateral rolled leaf mutant *url1t* and mapped the target gene on chromosome 1. A study done by Wang *et al*. (35) showed that a recessive gene *nal3* (*t*) responsible for narrow and rolling leaves was on chromosome 12. Zhou *et al.* (41) reported six recessive genes *rl1–rl6* that govern the "rolling leaf" trait which are identifiable via the Gramene web site (http://www.gramene. org/) situated on rice chromosomes 1, 4, 12, 1, 3 and 7. In addition they identified a rolling leaf mutant from *indica* variety Yuefeng B, named *rl11* (*t*), which shown reduced plant height, rolling and narrow leaves. A physical map encompassing the $rl_{11\ (t)}$ locus was constructed between SSR markers RM6089 and RM124 on chromosome 4 with physical position 29405123 to 34739636 and the target gene was finally defined to a 31.6 kb window between STS4-25 and STS4-26 on BAC AL606645. The beginning and end sequence of this BAC was used in a Blast search on the Rice Genome Annotation Project which reveals its position to be between 31629191 and 31817625 bp which covers 32 annotated genes. It is highly noteworthy that the physical position of EMMA4.2 in current study was 31746611 bp which is located between these two markers. These results revealed a particularly good agreement between these two studies. Thus it seems very likely that this QTL is the result of segregation of alleles of the $Rl_{11(t)}$ locus.

In this study differences in leaf rolling among rice cultivars has been revealed and QTLs identified. These data indicate areas of the rice genome containing genes of potential value in breeding drought resistant rice. Lists of positional and potential functional candidate genes are presented. These candidates included proteins which resemble those already described as having a role in drought resistance. These candidate genes are worth further investigation.

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رسم الخارطة الوراثية على مستوى الجينوم يكشف عن مواقع وراثية لصفات مقاومة الجفاف في محصول الرز(*Oryza sativa* L.)

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الملخص

أن هدف مربي محصول الرز هو تحسين مقاومة الجفاف في اصناف الرز ذات الحاصل العالي خصوصاً في المناطق المتأثرة من الجفاف، سيساعد تحديد مواقع الجينات التي تشارك في تجنب الجفاف على نحو كمي على استخدام هذه الجينات في تربية النبات والاسراع في الوصول الى هذا الهدف.اهتمت الدراسة الحالية بتقويم **328** صنفاً للرز التي تختلف فيها التفاف الأوراق، وقد استعملت طريقة (**EMMA**) **efficient mixed model analysis** مع اقل فرق معنوي ($p<0.0001$) لتحليل البيانات على اساس الصفات الكمية. كشفت نتيجة التحليل عن ثلاثة مواقع معنويه للصفات الكمية هي **EMMA4.2, EMMA6.2, EMMA7.1** على كل من الكروموسومات **4**، **6** و**7** على التوالي. وايضا تم الكشف عن الجينات المرشحة بصدد هذه المواقع بمسافة **200 kp** وكذلك تم تحديد الجينات الاكثر تعبيراً في انسجة الورقة وهي **Os04g53700**، **Os04g53930** **Os06g41770** التي قد تكون لها أهميه كبيرة في الدراسات المستقبلية.

جزء من اطروحة دكتوراه للباحث الأول.

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