Identification of quantitative trait loci for root depth in the rice Bala x Azucena F_6 mapping population using the buried herbicide method

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Abstract

Root depth is important for plant growth and survival during drought because of its role in water uptake from deep soil layers. The burying TRIK herbicide method has been applied to screen root depth in 138 recombinant inbred lines derived from crossing between two subspecies *Indica* Bala and *Japonica* Azucena. Analysis of mapping population revealed two putative QTLs on chromosome 6 near marker RZ682 and one on chromosome 7 near marker RG351 and two significant QTLs near marker G1010b and G1073 for root depth on chromosome 8. Positional candidate genes underneath QTLs were examined bioinformatically and through the literature revealing several interesting genes which may offer potential for developing drought resistant rice cultivars.

Key words: Rice, mapping population, buried herbicide, TRIK

*****Part of PhD thesis for the first author

تحديد الصفات الكمية ورسم الخارطة الوراثية لصفة عمق الجذور في الرز الى F_6 recombinant inbred line ناتج من التضريب بين Bala X Azucena باستخدام طريقة دفن مبيد الاعشاب

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ديفد ربنسون — **استاذ**

المستخلص

تم في هذه الدراسة استخدام طريقة دفن مبيد الاعشاب لتقيم F_6 recombinant inbred line **138 لصفة عمق الجذور ناتجة من التضريب بين** Balla x Azucena**. كشفت نتائج التحليل الوراثي عن عدة مواقع معنوية للصفات الكمية ، اثنان على كروموسوم رقم 8 قرب ماركر** b1010G **و1073**G **وموقع واحد على كروموسوم رفم 6 قرب ماركر**RZ**682 وموقع واحد على كروموسوم 7 قرب ماركر** RG**351. كذلك تم تحديد الجينات المرشحة لتلك المواقع التي ربما لها اهمية كبيرة في عملية استنباط اصناف من الرز مقاومة للجفاف.**

كلمات مفتاحية: **الرز، رسم الخارطة الوراثية، دفن مبيد الاعشاب، TRIK.**

*** جزء من اطروحة دكتوراه للباحث الأول.**

1. Introduction

In primary experiment the herbicide technique has permitted determination of variation in rooting as a function of origin, The differences in herbicide injury symptoms result from differences in the rate of root growth and the timing to reach the buried herbicide (Alshugeairy, 2013). Identifying loci and candidate genes controlling root traits using molecular mapping may aid in a better elucidation of the mechanisms responsible for differences in root depth and facilitate the development of markers for fast and cost effective breeding of rice with deeper roots by marker-assisted breeding.

A meta-analysis of QTLs for root traits in the Bala x Azucena population was reported by Norton *et al.* (2008) who focused on three rice root QTLs on

 ISSN 2072-3875

chromosomes 2, 5 and 9. The meta-analysis of these three QTLs has provided confidence intervals of 10.5, 9.6, and 5.4 cM respectively which contain 189, 322, and 81 genes. In another meta-analysis of QTLs on the Bala x Azucena population which focused on drought-related traits, compiled data from 13 experiments (Khowaja *et al*. 2009). meta-analysis of 24 published papers reporting root QTLs in 12 populations (Courtios *et al*. 2009), these papers on QTLs controlling 29 root parameters included root number, maximum root length, root thickness, root/ shoot ratio, and root penetration index, results for a total of 119 meta-QTLs were identified. Another QTL for the ratio of deep rooting *Dro1* (Deeper rooting 1) was mapped on chromosome 9 between the markers RM24393 and RM7424, the authors tested 117 recombinant inbred lines produced from a cross between IR64, a lowland cultivar with shallow rooting, and Kinandang Patong, an upland cultivar with deep rooting (Uga *et al*. 2011). Fine mapping was used by Sergeeva *et al*. (2006) to identify the *Cvi* allele of the *Inv* gene underlying a root elongation QTL in *Arabidopsis*, this was the first example of map-based cloning of a root QTL.

To date, few papers reported about cloning of root growth QTL genes in rice, Ding *et al*. (2011) investigated the phenotypic variations of near-isogenic lines (derived from crossing between Zhenshan 97 x IRAT109) under drought and normal conditions, the authors found that one of these lines (N19) contains qFSR4, a QTL on chromosome 4 controlling root volume per tiller related QTL and selected this position for fine-mapping due to the importance of this trait for drought resistance and their fine mapping resulted in three positional candidate genes of which *Narrow Leaf 1* is considered the most likely causal gene. The QTL *Dro1* was cloned in rice by Uga *et al*. (2012) who investigated this QTL in an upland field with drought stress and found that *Dro1* had a role in drought avoidance.

2. Materials and methods

2.1.Plant materials

Recombinant inbred lines (RILs) used for this experiment were derived by single seed descent from a cross between Bala (shallow rooted *Indica* cultivar) and Azucena (deep rooted *Japonica* cultivar) as described by Price *et al*. (2000).

2.2.TRIK herbicide used

TRIK herbicide active components as 46.7% w/w Diuron, 28 % w/w Aminotriazole and 14% w/w 2,4-D UK. In this study the TRIK herbicide dose was calculated based on previous experiments conducted by Roshi Shrestha where 100 mg per plant proved effective

2.3.Prepare pre germination of rice seeds

Seeds were surface sterilised in 1% sodium hypochlorite for 2 minutes then washed by soaking the seeds for a few minutes in beaker filled with tap water before being placed on a wet paper towel in a 9 cm Petri dish. Each Petri dish was sealed with Para film (Pechiney Plastic Packaging, Chicago) and incubated at 30^{o}C for 72 hours (Shrestha, 2008).

2.4.Experimental setup

The experiment was started in Cruickshank greenhouse on 21st of July 2010. As well as the experiment had 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. A total of 138 randomly selected RIL plus Azucena and Bala (140 altogether) were

ISSN 2072-3875

used. A box of 180 cm in length, 81.5 cm in width and 40 cm in depth was prepared using sides of plywood wrapped in thin plastic positioned over the asbestos sheeted bench. The bottom was covered with non-woven fabric which was folded up the sides to a height of 5 cm. containing loam top soil from Rolawn Ltd UK and herbicide were prepared as follows. The top soil used had a volumetric water content of 18.5%. To this 125 ml of nutrient solution per litre of soil was added to reach 27% water content. First, a 5 cm top soil layer saturated with Yoshida's full strength nutrient solution (Yoshida *et al*., 1976) was placed at the bottom. Above this was placed a filter paper soaked with TRIK herbicide (100 mg per plant was buried at 30 cm depth). Theta probes were placed at 30 and 15 cm depth (total of four theta probes used) see (Figure 1). The application of a herbicide score on a scale from 0 to 5 where 0 indicates no symptoms (Figure 2 A), score 1 indicates noticeable leaf yellowing (5-15% of leaf area affected) (Figure3 B), score 2 indicates substantial leaf yellowing (15-50% leaf area affected) (Figure 2 C), score 3 indicates substantial leaf yellowing (>50% leaf area affected) and noticeable leaf death (5-15% of leaf area) (Figure 2 D), score 4 indicates substantial leaf death (15-50% leaf area dead) (Figure 2 E) and score 5 indicates virtual to complete plant death (>50% leaf area) (Figure 2 (F). Symptoms of herbicide damage first appeared 20 days after sowing (DAS) and were recorded approximately every 4 days until 66 DAS. The soil water content started to reduce in the box at 6 DAS according to theta probe readings, after which water was added as required to maintain water content at 15 cm

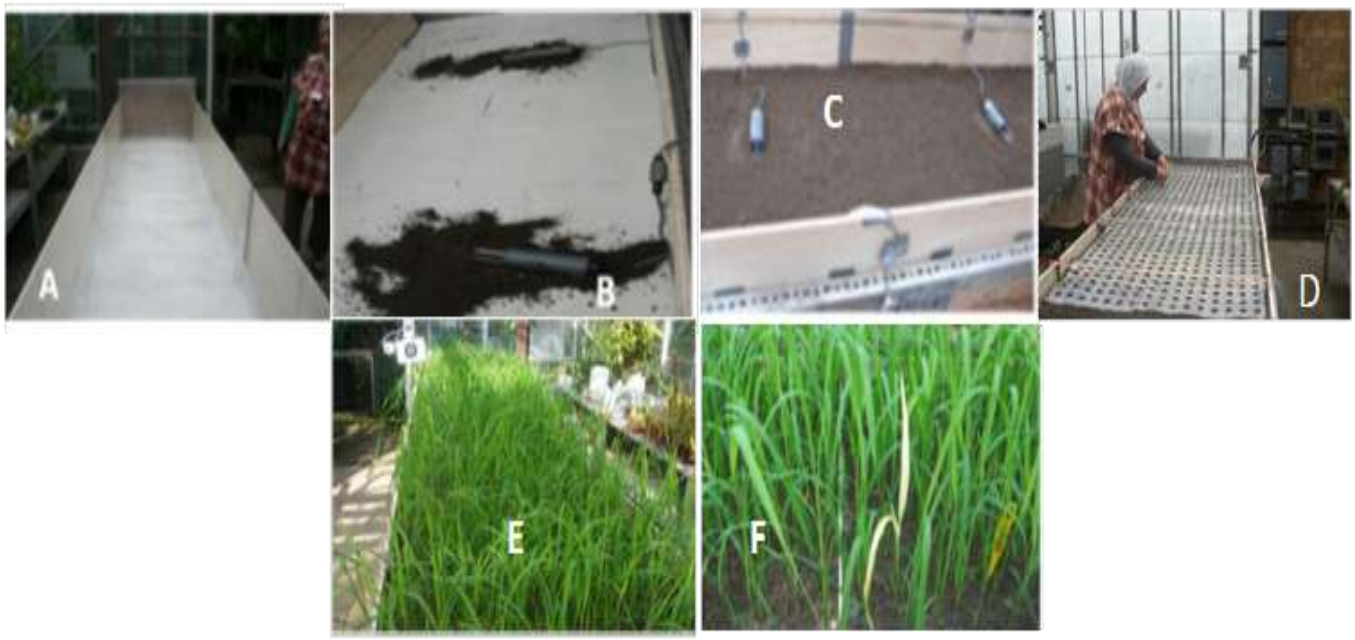

Figure 1: A; Above the saturated soil, a layer of filter papers was laid which had been soaked in 56g of TRIK in 300 ml of water and then dried; B; Above the filter paper was placed two theta probes; C; At depth 15 cm two theta probe was placed; D; The box was filled with top soil leaving 5 cm at the top and a plastic sheet containing perforations (2 cm diameter) for sowing plants at a 5 x 5 cm spacing was placed on top; E

ISSN 2072-3875

and F; Throughout the experiment a relative score of visible leaf symptoms from TRIK herbicide was recorded.

Figure 2: (A) Score 0 *(*no symptom)*;* (B) Score 1 (5 – 15% of leaf affected); (C) Score 2 (15 – 50% leaf area affected); (D) Score 3 (> 50% leaf area affected); (E) Score 4 (15 – 50% leaf area dead); (F) Score 5 (> 50% leaf area dead).

3. Statistical analysis

One-way (ANOVA) using Minitab version15 was carried to analyse the differences between the two parental Bala and Azucena varieties and between the RILs in herbicide damage score. The data were corrected for block effects and also for the normality by using Log_{10} base transformation before analysis

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4. Heritability computing

The broad-sense heritability (H^2) is the ratio of the total genetic variance to the phenotypic variance and refers to the degree to which genotype determines the trait (Price *et al*. 2002). The higher the value of H^2 for trait is the more suitable for the analysis of QTL mapping will be. According to Price *et al*. (2002) the formula (H^2) = 100*(F-1/F) was used to estimate the broad-sense heritability, the F-values is the analysis of ANOVA statistic.

5. Detection QTL position

The marker data for the population was last updated in 2007 and has 164 markers (Norton and Price, 2009). QTL detection was by composite interval mapping (this is a precise method to identifying the actual genes underlying QTLs of the desirable trait as mentioned in part

ISSN 2072-3875

1.7.3) and employed the programme QTL Cartographer, version 1.15 (by Basten; Wei and Zeng, Department of Statistics, N. Carolina State University) as reported in Price *et al*. (2002). By using the same programme, permutation analysis (1,000 permutations) for these data suggested that a LOD score of 3.2 is useful as the genome-wide 5% significance threshold. Putative QTLs between LOD scores of 2.5 and 3.2 are considered worthy of reporting since they might well be real QTLs.

6. Analysis of candidate genes

A list of positional candidate genes 2 cM either side from the peak QTL position were gathered as described in Price (2006) and are presented as a list (ALshugeairy,2013). Relating each marker to the physical position in the genome is described in Khowaja *et al*. (2009) where a supplementary table provides the base pair position for each marker. Gene lists on Psuedomolecules 6.1 were downloaded from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). The chosen candidate genes were positional candidates as they were situated near the region of the QTL detected. All the positional candidate genes were examined to verify if they have full-length cDNA (fl cDNA) and expressed sequence tag (EST) listed in the genome browser using the website Rice Genome Annotation Project. In addition, individual genes were examined for gene expression in plant tissue by using the rice expresion profile database (RiceXPro) http://ricexpro.dna.affrc.go.jp/ (Sato *et al*., 2011) 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 (ALshugeairy,2013). The candidate genes with root tissue expression were further examined through the literature to see whether they related to cell expansion or root elongation in other studies to reduce the number of candidate genes.

7. Results and discussion

The mean of theta probe reading against the date is presented in Figure 3. The soil water content at 15 and 30 cm depth was maintained over 20% and 30% respectively indicating continuously moist conditions until the end of the experiment. A dot plot of the herbicide scores as the experiment progressed is presented in Figure 4, while a mean value and the value of F from the one way ANOVA is presented in Figure 5. The symptoms of herbicide damage were first recorded for the population at day 20, when only 14 genotypes had symptoms and the mean value of the herbicide score was only 0.02. Even at this point there were strong differences between genotypes in the herbicide score ($P < 0.001$) which increased in significance from 20 day after sowing (DAS) to 66 DAS. These scores indicated that, RIL4, RIL196 and RIL162 had high scores while RIL73, RIL 76, RIL 90, RIL 126, RIL 182, RIL 205, RIL 220, RIL 226, RIL 237, RIL 256, RIL 265, RIL 266 and RIL269 did not show any symptoms even by the end of the experiment. The mean of herbicide score and F value increased with the time from day 20 after sowing and the majority of plants were affected by the end of this period. The F value (and hence the broad sense heritability) peaked at 59 days indicating that this is the period with the highest discrimination between genotypes and this is therefore the value that will be used for QTL mapping. The mean of the Bala and Azucena (parental) and mapping population values of herbicide score per plant at 59 days are given in Table 1. Azucena had nearly double the herbicide score of Bala ($P < 0.05$). The mean values for F_6 mapping population were higher than Bala and less than that for Azucena. The frequency distribution of herbicide score displayed a wide range of variance and transgressive segregation (Figure 6). In addition, the heritability of herbicide score trait was 76%.

ISSN 2072-3875

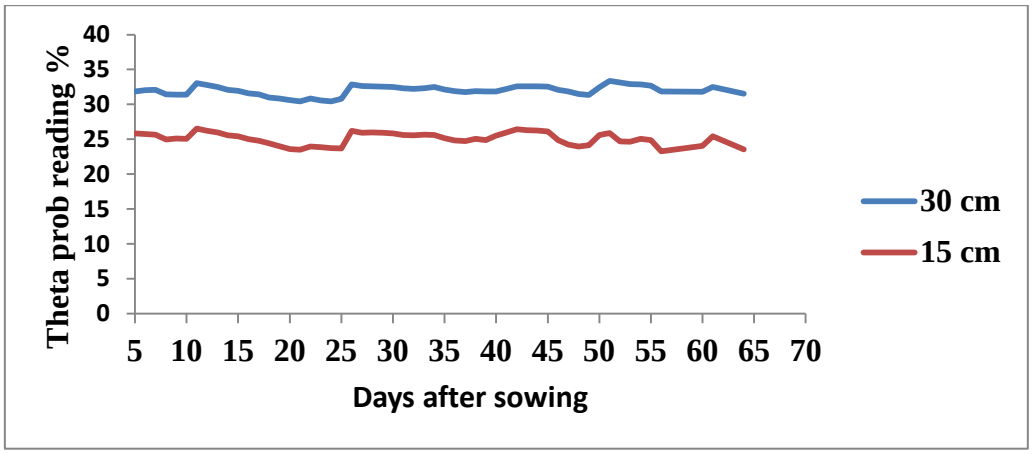

Figure 3: The average theta probe reading against the days after sowing with two different depths 15 cm with red line and 30 cm with blue line.

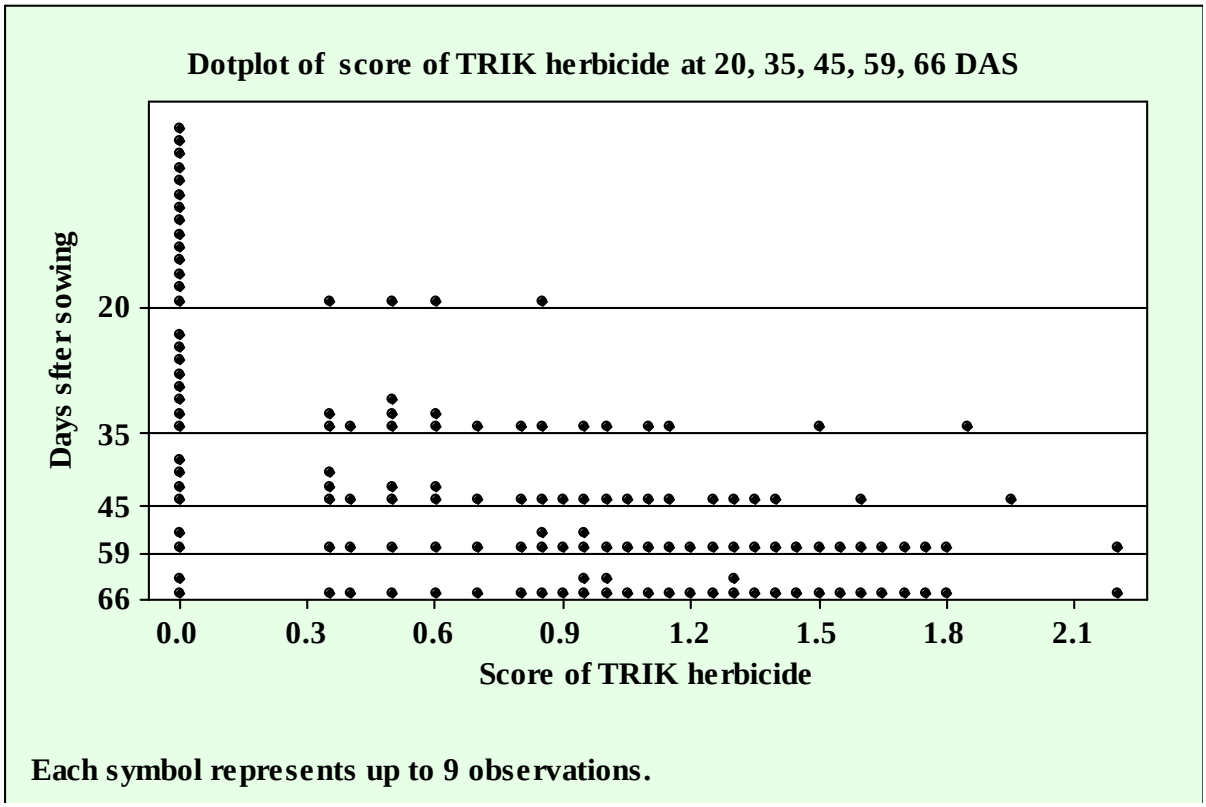

Figure 4: Dotplot of TRIK herbicide score at different days.

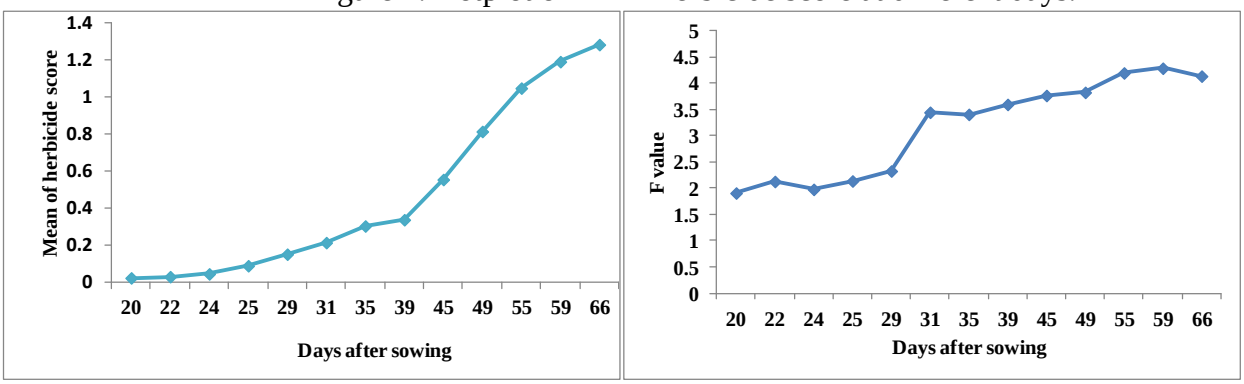

Figure 5: Mean of herbicide score (Left) and F value from one-way analysis of variance testing differances among RILs (Right) during the course of the experiment.

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Table 1: Mean herbicide score of Azucena and Bala with ± standard deviation and broad sense heritability (H^2) in the mapping population and the parental lines in response to TRIK herbicide

Traits	*n*	Score herbicide/plant (59 DAS)
Azucena	4	2.1 ± 0.2
Bala	4	1.1 ± 0.8
F_6	138 × 4	1.2 ± 0.8
h^2 (%)		76

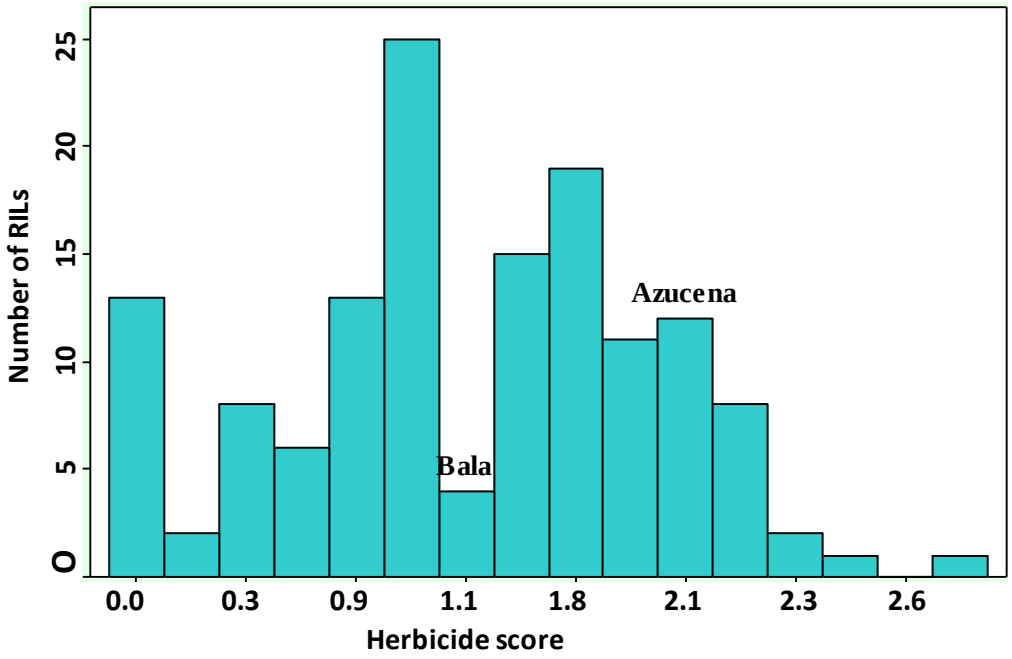

Figure 6: The frequency distribution of herbicide score of F6 mapping population and parents at 59 DAS.

7.1 QTL detection

A detail of the QTL analysis is presented in Table 2. A total of two QTLs for herbicide score was detected on chromosomes 8, one putative QTL on chromosome 6 and one putative QTL on chromosome 7 (Figure 7).

Table 2: QTLs controlling root traits in rice based on composite interval mapping using RILs generated from the cross between Bala and Azucena. R^2% is the proportion of the total phenotypic variance explained by the QTL

Chromosome	**Distance in cM from nearest marker**	**LOD scor**	**Donor of +ve allele**	***R*^{2} %**
6	RZ682+4	3.05	Azucena	6.43
7	RG351+3.6	3.13	Bala	5.37
8	G1010b	8.71	Azucena	16.79
8	G1073+6.8	9.26	Bala	24.53

ISSN 2072-3875

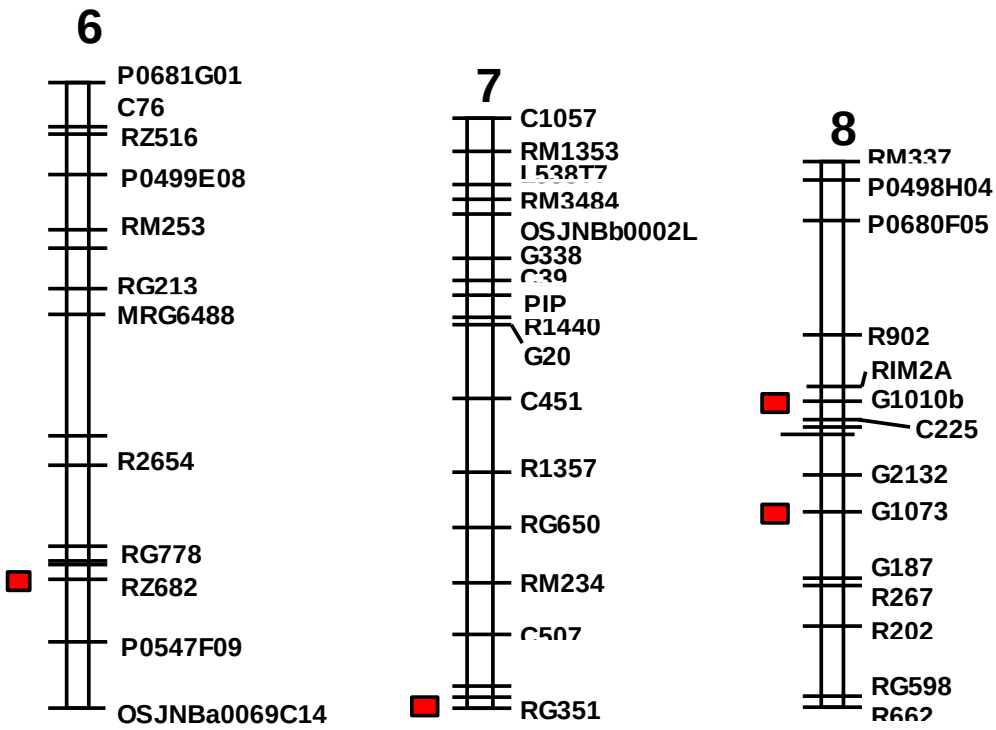

Figure 7: Main effect QTLs detected by composite interval mapping (in QTL-Cartographer) for herbicide score at 59 DAS. The QTLs are drawn as boxes, where each box represents the one- LOD confidence intervals**.**

7.2. Potential candidate genes

The conventional approach for potential candidate genes is to search within one LOD confidence interval either side of the peak position. However, Price (2006) reported that when QTLs were fine mapped or cloned they tend to fall within 2 cM of the original mapped position and this justification came from a review of 20 plant QTL studies. For that reason, when attempting to identify candidate genes underneath QTLs, it is considered appropriate to include genes within 2 cM either side of the detected QTL peak.

In current study it was interesting to identify candidate genes underneath QTLs which have the positive donor allele inherited from Azucena so in this thesis those from Bala were not considered further. The putative QTL on chromosome 6 was at 138 cM on Bala x Azucena map. There are roughly 63 genes within 2 cM of this position (ALshugeairy,2013). Of these, 27 genes did not show any expression in plant tissue according to the RiceXPro database, 35 genes were expressed in different part of plant tissue in root tissue and one gene was expressed in plant tissue but not in root tissue. In addition for all these 35 genes their annotation terms were searched in the literature. Three standouts are Histidine kinase (*AHK*) (LOC_Os06g44410)**,** AP2 domain containing protein (LOC_Os06g44750) and auxin efflux carrier component, putative, expressed (LOC_Os06g44970). Chromosome 8 had one QTL from Azucena at 64 cM near marker G1010b with physical position (7084938 - 7146705) bp. A total of 183 candidate genes were detected from the QTL position 2 cM interval (6.83 - 9.26 Mb) on the genome (ALshugeairy,2013). Of these, a total of 105 genes do not appear to be expressed in plant tissue, 77 genes were expressed in different part of plant tissue and and in root tissue and one genes were expressed in plant tissue but not in root tissue. A total of two annotated genes were selected from those expressed in roots which are villin, putative, expressed (LOC_Os08g14230) and ADP-ribosylation factor

ISSN 2072-3875

(LOC_Os08g15040)). From a list of genes related to root length which tested by using RiceXPro http://ricexpro.dna.affrc.go.jp/

In primary experiment, it is clear that buried TRIK herbicide can cause significant symptoms in plants grown in soil-filled boxes under greenhouse conditions. Previously, it was observed that Azucena had a higher herbicide score than Bala and this was confirmed from the results from this study. This observation suggested a logical step to test herbicide score in the RIL population of Bala × Azucena in order to map QTLs controlling the genetic variation between Bala and Azucena and thereby confirm the value of the method for high throughput screening of root depth. Herbicide score proved to be highly heritable. The frequency distribution of herbicide score in the mapping population showed a wide range of variation indicating the occurrence of transgressive segregation (Figure 7). Some RILs had higher herbicide scores than Azucena and some less than Bala, indicating that alleles from both parents contributed to the herbicide score. The data suggest that it might be possible to breed varieties with greater herbicide score than that seen in Azucena.

The Bala x Azucena population has been screened for root traits in many different media and the results summarised in Khowaja *et al*. (2009). A heat map of root QTLs in that paper shows that the two regions highlighted in this herbicide study are weakly active for root QTLs. On chromosome 6 near RZ682 QTLs have been detected for the root length in soil (Price and Courtois, 1999), number of roots (Price *et al*., 2000), root mass in rhizotrons (Price *et al*., 2002), root mass and root thickness in soil boxes (MacMillan *et al*., 2006) and root thickness in hydroponics (Price unpublished data). On chromosome 8 near marker G1010b, QTLs have been detected for root penetration (Price *et al*., 2000), number of roots at depth in rhizotrons (Price *et al*., 2002), root thickness in the field (Renee Lafitte unpublished) and in rhizotrons, and root length in hydroponics (Price unpublished). Price *et al*. (2002) reported that the region near marker RZ682 on chromosome 6 affected the number of roots past 100 cm and root thickness in rice populations generated from a cross of 1R58821-23-B-1-2-1 x 1R5261-UBN-1-1-2 (Ali *et al*., 2000). In addition, the region near marker G1010b on chromosome 8 was affected deep root weight and number of roots past 100 in the Azucena x IR64 population (Hemamalini *et al*., 2000). Hence the QTLs determined here may be valuable in breeding for sustainable varieties with high production even in the presence of drought.

(ALshugeairy,2013) it had been chosen these five candidate gene being reported according to their putative functions in literature done by others.

Examining the RiceXPro database of gene expression and literature for genes around the QTL on chromosomes 6 and 8 resulted in five candidate genes that might be responsible for root elongation in rice. Identifying candidate genes will advance our understanding of the processes involved in expansion and elongation of cells in rice. They are detailed below:

Histidine kinase (*AHK*) (LOC_Os06g44410), is a family of genes which consist of *CKI1, AHK1 (AtHK1), AHK2, AHK3, CRE1 (AHK4, WOL1), CKI2 (AHK5, ETR1, ERS1, ETR2, EIN4, ERS2, PHYA, PHYB, PHYC, PHYC*. This candidate gene has high expression intensity in root tissue of roughly 2200 Cy3 (Fluoresce yellow-green Cyanine dye) in RiceXPro. A study done by Ueguchi *et al*. (2001) reported that a total of three novel genes had been identified in *Arabidopsis thaliana*, which are AHK2, AHK3 that possibly function in the plasma membrane and *AHK4* mainly expressed in roots. Nishimura et *al*. (2004) reported that *AHK* genes are expressed ubiquitously in various tissues during postembryonic differentiation and development. The authors proposed that the main functions of *AHK* genes and those of endogenous cytokinins are initiating the division of the cell and sustaining the competence of the meristematic cells to avoid subsequent differentiation until a sufficient number of cells have accumulated during organogenesis.

Another candidate gene is **AP2 domain containing protein (LOC_Os06g44750).** This is a huge family containing 144 AP2 domain genes, which are divided into five subfamilies (Sakuma *et al*., 2002). The AP2 subfamily contains two AP2 domains connected by a conserved linker. This gene had high expression in root tissue of about 2000 Cy3. These genes are transcription factors. The most similar Arabidopsis gene is a AP2/EREPB transcription factor At5g57390

 ISSN 2072-3875

which has a putative role in root development as well as many other things (www.Arabidopsis.org). **Auxin efflux carrier component (LOC_Os06g44970)** is part of the PIN family of genes (***PIN1- PIN18*****). This candidate had high** expression **in root tissue that reaches approximately 15000 Cy3**. Lee and Cho (2006) reported that *PINFORMED3* (*PIN3*, *PIN3ox*) are specifically involved in the root hair cell and mediates the root gravitropism. Qi, *et al*. (2012) suggested that auxin has an important role in generating the optimal root system architecture that can cope with growth reductions of crops induced by water or nutrient shortages. Auxin acts as a versatile trigger in root developmental processes (Benkova *et a*l., 2009), including the regulation of root growth (Ding and Friml, 2010), root patterning (Friml *et al*., 2002) and root cell division and elongation (Mironova *et al*., 2010). Importantly, auxin transporters, including PINs, have been speculated to be underlying the three biggest root growth QTLs in this mapping population (Norton *et al*., 2008).

Villin, putative, expressed (LOC_ Os08g14230), using the database RiceXPro showed that this gene is of high expression intensity in root tissue that reach about 6000 Cy3. The role of actin filament bundles in all plant cells is to serve as tracks for myosin-dependent organelle movement and organization of the cytoplasm (Thomas, 2012). The role of the actin-bundling protein villin was investigated by Van der Honing *et al*. (2012) in Arabidopsis (*Arabidopsis thaliana*). The authors produced a double mutant in which *VILLIN2* (*VLN2*) and *VLN3* transcripts are truncated. This caused local differences in cell length and led to a twist in different parts (leaves, stems, siliques, and roots) of the *vln2 vln3* double mutant plants. The authors believed that this gene functions in the generation of thick actin filament bundles, which serve in the regulation of coordinated cell elongation.

ADP-ribosylation factor (LOC_Os08g15040), named as ARFs are subfamily of the Ras superfamily of GTP-binding proteins (Gebbie *et al*., 2005). This gene has been shown to be highly expressed in root tissue with about 49000 Cy3. A study by Yoo *et al*. (2008) reported that AGD1, a class IADP-ribosylation factor GTPase-activating protein, is important for maintaining straight growth in Arabidopsis root hairs, since mutations in the AGD1 gene resulted in wavy root hair growth.

8. Conclusion

On the basis of the results obtained from this study we suggest that QTL mapping of TRIK herbicide score in F_6 mapping population derived from Bala x Azucena confirms the fact that Azucena has a deeper root system than Bala. The mapping results revealed two significant QTLs located on chromosomes 8 and two putative QTLs on chromosomes 6 and 7. These QTLs coincide with regions of QTL activity in the same mapping population, even if these were not the most active regions normally detected. The region 2 cM either side was investigated for presence of candidate genes related to expansion and cell elongation with reference to the rice genome sequence. Positional candidate genes were selected based on their expression pattern in rice tissue, their putative function listed on the Rice Genome Annotation Project and literature searching. The five candidate genes selected were revealed as a perfect candidate for future studies, including a PIN auxin transporter which is considered particularly noteworthy

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