

Rice Diversity Panel Mapping for Identifying Genetics Behind Leaf Vein Density Trait in Rice (*Oryza sativa* L.)

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Abstract: A screen of rice genotypes was carried out on a total of 327 genotypes of the rice diversity panel for leaf vein density. Identifying locations of genes that impart to leaf vein density in a quantitative way should capable the using of these genes in plant breeding and accelerate producing C4 rice plant. Finding of genotypes of rice that have less distances between veins is still the challenge of the plant breeders. Screen was shown significant variations in leaf vein density. Quantitative trait loci (QTLs) for the leaf vein density trait were identified by using Efficient Mixed Model Analysis (EMMA). QTLs were considered reportable if they had *P* values (below 0.0001). The most significant Single nucleotide polymorphisms (SNP) associations (EMMA 1.3, EMMA 1.8 and EMMA 10.1) were in each of the rice chromosomes 1 and 10 respectively. All genes positioned 200 kb around associations were selected. The candidacy of the most promising were NADP-dependent malic enzyme, chloroplast precursor (LOC_Os01g09320; ras-related protein, putative, expressed (LOC_Os01g51700); 60S acidic ribosomal protein (LOC_Os01g09510); Auxin-responsive Aux/IAA gene family member (LOC_Os01g09450; myb-related transcription activator, putative, expressed (LOC_Os01g09280); glycine-rich protein (LOC_Os01g09246); phosphofructokinase (LOC_Os01g09570); oxidoreductase, short chain dehydrogenase/reductase family (SDR) LOC_Os10g31780), which have been expressed in leaf tissue and requisite to be investigated further.

Keywords: Rice Diversity Panel, Rice, Leaf Vein Density, Mapping

1. Introduction

To meet the ever growing world population, it is necessary to increase yield that can only be done by increasing the efficiency with which photosynthesis uses solar energy [1, 2]. Plants are differing in canopy photosynthesis that leads to differences by about 50% between plants in the radiation use efficiencies [3, 4]. C4 crops, grown in hot and dry environments, have higher yields, reduced water loss and increased nitrogen use efficiency compared to C3 crops such as rice. Plants use three photosynthetic pathways [5]. Plants that are called C3 produce a three-carbon compound by using photosynthetic pathway in which CO₂ is fixed by Ribulose Biphosphate Carboxylase/Oxygenase (RuBisCO) in the Calvin-Benson cycle. Whereas species, that evolved from C3 plants, use the C4 and Crassulacean Acid Metabolism (CAM) pathways, and in both cases, a four-carbon compound is initially formed from fixation of HCO₃. It is suggested that C3 is the ancestral pathway. Thus, C4 and CAM are forms diverged from C3 plants in recent times. The majority of C4 plants, and certainly all known C4 grasses, compartmentalize

photosynthetic reactions between two morphologically distinct cell types that are located in circles around veins [6]. There are enlarged bundle sheath (BS) cells around the veins that are surrounded by mesophyll (M) cells that called Kranz anatomy. This structure produces a consistent interveinal distance of four cells (vein-BS-M-M-BS-vein). Each of BS and M cells shared metabolic reactions such that C4 acids are generated in M cells and then diffuse to the BS where the Calvin-Benson cycle operates. Restricting the expression of a few numbers of genes to either the BS or M cells achieves this separation of metabolism. [7] reported that Carbonic anhydrase (CA), phosphoenolpyruvate carboxylase (PEPC), NADP-malate dehydrogenase (MDH), pyruvate orthophosphate dikinase (PPDK) and the proteins involved in their post-translational regulation accumulate in the M cells, while NADP-malic enzyme (ME) and RuBisCO are restricted to the BS. All of these enzymes encoded by genes that are present in C3 plants, but the levels of expression are much lower than in C4 species. According to the polyphyletic evolution of the C4 pathway, the transition from C3 to C4 seems to be relatively simple. the most obvious differences between leaf morphology in C3 and C4 plants is

leaf venation pattern indicating that veins play an important role in the differentiation of C4 leaf anatomy. Vein density measured in a range of C3 and C4 species demonstrated that veins are consistently more closely spaced in C4 species [8]. In addition, quantitative measurements of BS-to-M cell ratios in C3 and C4 leaves showed that in C4 plants the ratio approaches 1:1 [9, 10, 11]. This ratio equates to veins (V) being separated by only four photosynthetic cells in C4 leaves as opposed to up to 20 cells in C3 leaves. As such, the repeating V-BS-M-M-BS-V unit of Kranz anatomy is produced. One remarkable exception to this repeating model is found in *Arundinella hirta*, a C4 grass that exhibits an atypical anatomy where wreaths of so-called distinctive cells are found between V-BS-M-M-BS-V units [12, 13]. The distinctive cells carry out the same function as BS cells but are not themselves associated with veins [14, 15, 16]. Notably, if the number of BS and distinctive cells is combined, the 1:1 ratio is also observed in *Arundinella hirta* (17) reported that a comparison of vascular development in C3 and C4 Flaveria species showed that both the major and minor veins were commenced at comparable stages in development but that a higher number of minor veins were commenced in the C4 species. An acquisition of a mechanism to induce procambium at more regular intervals across the leaf may be the first step in the evolution of Kranz anatomy. The role of auxin in vascular development is recognized, it is probably that such a mechanism was adapted from existing auxin pathways. A study that compared anatomical and biochemical differences between 16 Flaveria species that included C3, C3-C4 intermediate, C4-like, and C4 types gave more evidence for the suggestion that changed vein patterning was an early modification in the evolution of C4. To find accessions of rice with less interveinal distance is the challenge for plant breeder. To determine the extent to which rice leaf morphology can vary, it is important to screen rice genome wide association for leaf morphology to determine variations in interveinal distance.

2. Material and Methods

The layout of this experiment was randomized complete block design with four replications. A subset of 327 accessions was utilised for this study. Of those examined, 279 belonged to the rice subpopulations *aromatic* 9, *aus* 53, *indica* 57, *temperate japonica* 75 or *tropical japonica* 85. The other 48 accessions were grouped as admixtures between subpopulations. This experiment used box (length 450, width 90 and depth 40 cm) filled with loam top soil from Rolawn Ltd (UK). A plastic sheet (450 x 90 cm) was placed on the top of the soil (soil surface). This plastic sheet with 1308 perforations (2 cm diameter) for sowing rice plants at depth 1.5 cm using 5 x 5 cm spacing was made. On 2013, 327 rice diversity panel accessions were sown. For 12 hours a day, supplementary light of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ Photosynthetically active radiation (PAR) was supplied within temperature range from 28 - 30°C and watered with Yoshida's full strength nutrient solution [18]. Vein counts were made off-site at green house - University of Aberdeen from images snapped of freshly collected leaves. Veins images in the second completely expanded newly harvested youngest leaf at 8

weeks after sowing were captured for screening the 327 rice diversity panel by a digital camera model canon (EOS 7D Mark II DSLR Camera, 20.2 MP). Veins were counted at the widest area of the leaf including the midrib or edges (Figure 1).

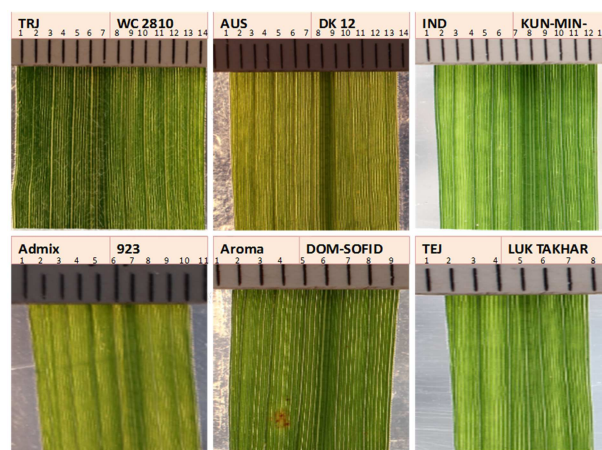


Figure 1. Images of leaf vein of different rice subpopulation (*indica* (IND), *aus*, *aromatic* (Aroma), *tropical japonica* (TRJ) and *temperate japonica* (TEJ)) at 8 weeks after sowing. Scale in millimetre.

Genotyping of the Rice Diversity Panel has been done at Cornell University, New York on an Affymetrix genotyping array which includes of 44,100 SNPs distributed over the rice genome (380 Mb) [19]. For this SNP chip, the estimation was about 1 SNP/10 kb coverage [20]. The association mapping analysis was done by Dr. Alexander Douglas Statistician and Bioinformatician (University of Aberdeen). An Efficient Mixed Model Analysis (EMMA) accounting for population structure was performed on all the genotypes according to methods reported by [21] that modified from [22] who developed a novel mixed-model method to simultaneously take into account for many levels of relatedness identified by random genetic markers. In addition, this analysis with EMMA plus separate sub-populations is the same to the statistical method adopted by [19] in the first publication mapping traits using this SNP data on this population. The result presented by Dr Douglas provided were many files containing 4 pdf files (Histogram, Manhattan mixed, Manhattan naïve and QQ plot). In addition, the statistic for the minor allele frequency value that was taken from previous analysis of other data was performed, and has relation with the allele of the SNP that is of lowest frequency within the genotypes. The SNP was considered potentially not reliable, when this proportion was less than 0.05. Furthermore, analysis of the separate subspecies was done; the aromatic and admixtures were removed from the data set prior to association mapping analyses as there were insufficient numbers of those within these subspecies. As well as, the four subspecies of association populations were analysed for association mapping. It's important to mention that the literature proposes that there is no uniform threshold *P* value that can be considered in rice diversity panel [19]. In current study, 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%. Using the

method of [19], genes positioned approximately 200 kb around associations (excluding transposons) were considered positional candidates (assuming linkage disequilibrium of 200 kb). Consequently, lists of genes within this location were collected via the rice Pseudomolecule version 6 from the rice genome annotation scheme. So in order to collect more data about candidate genes, the expression pattern of each was evaluated bioinformatically via the database of rice expression profile (RiceXPro) that can be found at <http://ricexpro.dna.affrc.go.jp/> after converting Rice Genome Annotation Project (RGAP) names to International Rice Genome Sequencing Project (IRGSP) titles at <http://rapdb.dna.affrc.go.jp/tools/converter/run>. Furthermore, the candidate genes with clear expression in leaf tissue were considered further in the literature to decide whether they are related to cell growth or leaf vein development in other studies which would nominate them excellent candidate genes.

Statistical and bioinformatic analysis

All the data were analysed using Minitab version 15. Two-way ANOVA with factors genotype and block was applied. The data were corrected for the block effect and also for normality by using base \log_{10} . Using EMMA, the association mapping analysis was made. The reliability of Trait-marker associations was with P values below 0.0001. The significant SNPs were tested for minor allele frequencies, with values above 0.05 considered to be reliable. All genes located with 200 kb of the QTL identified were selected as positional candidates. To distinguish between genes and likely pseudogenes, all the candidate genes have been tested for full-length cDNA (fl, cDNA) and expressed sequence tag (EST) [23]. In order to test gene expression in plant leaves, the database of RiceXPro at <http://ricexpro.dna.affrc.go.jp/> was used [24]. Furthermore, the candidate genes with significant expression were examined for whether they are linked to leaf vein density or even to cell development through literature studying to decrease the number of candidate genes.

3. Results and Discussion

Leaf vein density was assessed at eight weeks after sowing. The output of One-way ANOVA showed that there were high significant differences in leaf vein density between the genotypes ($F = 2.71$, $P < 0.001$, $R^2 = 30.03$). The genotypes NOVA, BJ 1, WC 4419, HATSUNISHIKI, LD 24, GEUMOBAYEO, LEUANG, HAWN, SL 22-613 and WIR 3039 had the highest value for leaf vein density while the genotypes Pirinae 69, Lomello, 9524, Halwa Gose red, SUFAID, GOTAK GATIK, Italica Carolina, LACROSSE, WC 6 and VICTORIA F. A had the lowest value (Figure 2). Figure 3 presents the cumulative distributions of P values in a rice diversity panel scan for leaf vein density again showing the value of the mixed model in controlling false positive associations. Association analysis of the leaf vein density is presented graphically in Figure 3. Use of EMMA revealed that three SNP are significantly associated with leaf vein density using a threshold value from the association analysis of under 0.0001 ($-\log_{10} P = 4$) (Figure 4, Table 1). The most significant SNP association were EMMA 1.3, EMMA 1.8

and EMMA 10.1 with minor allele frequency are 0.40, 0.37 and 0.46 respectively which indicates that this association is reliable. From association analysis for individual rice groups *aus*, *indica*, *tropical japonica* and *temperate japonica* with leaf vein density were detected (Figure 5). In the present study only those SNPs detected in the mixed model have been taken forward for listing candidate genes.

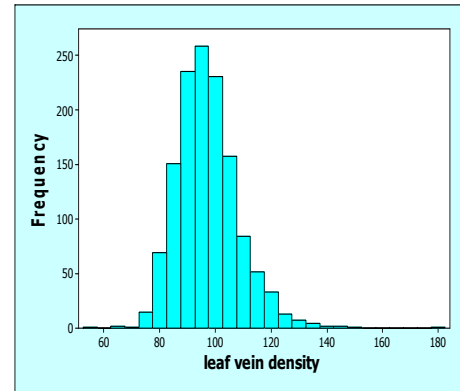


Figure 2. Histogram of leaf vein density for different rice genotypes at 8 weeks after sowing.

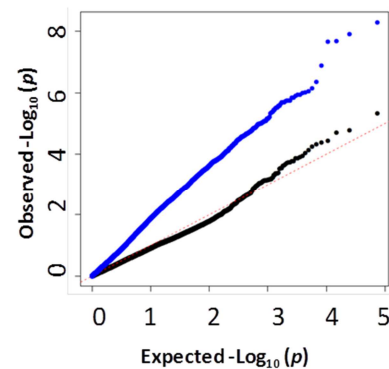


Figure 3. Cumulative distributions of P -values in rice diversity panel scan for leaf vein density. The upper dotted line is the naïve data plot, lower dotted line is the data corrected for population structure using efficient mixed-model association (EMMA). The faint dotted line is a $y = x$ plot.

Table 1. seventy six associated SNPs with rice diversity panel significance for leaf vein density.

Association name	Chromosome	SNP id	Position (bp)	$-\log P$
EMMA1.1*	1	id1003850	4649725	2.741437
EMMA1.2	1	id1003922	4754473	4.431066
EMMA1.3	1	id1003932	4816430	4.76643
EMMA1.4	1	id1003946	4824928	3.125417
EMMA1.5	1	id1003966	4869563	3.768984
EMMA1.6	1	id1003969	4870786	4.027962
EMMA1.7	1	id1015694	26923741	3.216116
EMMA1.8	1	id1017799	29870072	5.316268
EMMA1.4	1	id1017800	29870105	3.931617
EMMA1.10	1	id1018186	30259251	3.862305
EMMA1.11	1	id1018208	30277466	3.852993
EMMA1.12	1	id1018264	30372127	2.983439
EMMA1.13	1	id1019252	31733901	3.137514
EMMA1.14	1	id1019318	31810076	3.112504
EMMA1.15	1	id1019442	31975536	4.703504
EMMA1.16	1	id1019599	32098049	2.88368
EMMA1.17	1	id1019921	32417755	4.245419
EMMA1.18	1	id1019965	32448978	3.776631
EMMA1.19	1	id1020064	32537533	3.548692
EMMA1.20	1	id1020172	32657373	3.383683

Association name	Chromosome	SNP id	Position (bp)	-logP
EMMA2.1	2	id2010961	25248042	2.961937
EMMA3.1	3	id3002904	4870549	3.092584
EMMA3.2	3	id3002906	4870903	3.141756
EMMA3.3	3	id3002970	4984657	3.039798
EMMA3.4	3	id3011375	27044188	3.161961
EMMA3.5	3	id3011400	27088843	3.140736
EMMA3.6	3	id3011406	27094275	3.143558
EMMA3.7	3	id3011485	27274022	3.312589
EMMA3.8	3	id3011493	27278957	3.371414
EMMA3.9	3	id3011506	27304002	3.361587
EMMA3.10	3	id3011582	27382750	2.872733
EMMA3.11	3	id3011726	27463661	3.071912
EMMA3.12	3	id3016016	33093386	2.777638
EMMA3.13	3	id3018359	36129563	3.148368
EMMA4.1	4	id4001786	4317452	2.899073
EMMA4.3	4	id4006437	20381599	2.776798
EMMA6.1	6	id6004216	6599402	3.055338
EMMA6.2	6	id6011598	22312304	3.17969
EMMA6.3	6	id6011606	22316719	3.176603
EMMA6.4	6	id6014187	26188962	3.016867
EMMA6.5	6	id6016050	27844031	3.363608
EMMA6.6	6	id6016215	28383507	2.965974
EMMA7.1	7	id7004157	23422997	3.153137
EMMA8.1	8	id8004945	18672697	3.680596
EMMA9.1	9	wd9001498	10122677	2.871118
EMMA10.1	10	id10004603	16614044	4.379868
EMMA10.2	10	id10004606	16615168	4.32307
EMMA10.3	10	id10004614	16619029	3.736666

*EMMA1.1 refers to the method by which the rice diversity panel was analysed, chromosome number, the SNP order within each chromosome. The highly significant associations are in bold.

Table 1. Continued seventy six associated SNPs with rice diversity panel significance for leaf vein density

Association name	Chromosome	SNP id	Position (bp)	-logP
EMMA10.4	10	id10007116	22476823	3.121138
EMMA10.5	10	id10007134	22494504	2.938758
EMMA10.6	10	id10007137	22497992	3.722401
EMMA10.7	10	id10007143	22502368	3.900588
EMMA10.8	10	id10007152	22503803	3.711377
EMMA10.9	10	id10007153	22504612	3.012452
EMMA10.10	10	id10007165	22576427	3.524363
EMMA10.11	10	id10007166	22576547	3.625706
EMMA10.12	10	id10007167	22576599	3.313808
EMMA10.13	10	id10007176	22611907	4.127244
EMMA10.14	10	ud10001291	22648621	2.859625
EMMA11.1	11	dd11000503	21975605	3.617917
EMMA11.2	11	dd11000982	22148499	3.050962
EMMA11.3	11	dd11000986	22148852	2.713268
EMMA11.4	11	dd11000988	22148992	2.702486
EMMA11.5	11	dd11000989	22149066	3.07144
EMMA11.6	11	dd11000992	22149249	2.714034
EMMA11.7	11	dd11000994	22149315	3.178163
EMMA11.8	11	id11008642	22149608	2.908412
EMMA11.9	11	id11008863	22558101	3.241729
EMMA11.10	11	id11008875	22610675	2.956577
EMMA11.11	11	id11008887	22616701	2.831769
EMMA11.12	11	id11008894	22648493	3.159423
EMMA12.1	12	id12007710	22740633	2.74965
EMMA12.2	12	id12009451	25871148	2.956367
EMMA12.3	12	id12009470	25890219	3.045969
EMMA12.4	12	id12009931	27171774	3.533409

*EMMA1.1 refers to the method by which the rice diversity panel was analysed, chromosome number, the SNP order within each chromosome. The highly significant associations are in bold.

Assessing of vein density of leaf in 327 rice accessions considered as a long term aim of introducing the mechanism of C4 into C3 plants. It was possible to show that there was significant variation across genotypes perhaps reflecting differences in the degree of the close relation between development of the vein and leaf width is associated to fracture proliferation in stretched material that leads to auxin-like signals in the new spaces in which new veins can be formed. Consequently, the link connecting the formation of auxin and vascular might explain the link between vein density and width of leaf [25]. In addition, the substantial variation in leaf vein density among genotypes was indentifying in current study which is confirmed by [25] who reported that there is variation in vein density, also by examining two rice mutants, they found that the trait of narrow leaf width was related to a high vein density trait but there was incomplete linkage. However more studies need to be done in order to exploit data above to set up future investigation. In addition, all genes situated 200 kb around associations, that include candidate gene lists detected by using the EMMA approach, were chosen (Table 1). The RiceXPro database shows that many candidate genes are expressed in leaf tissue. From these, eight stand out after seeking gene function in the literature (Table 2).

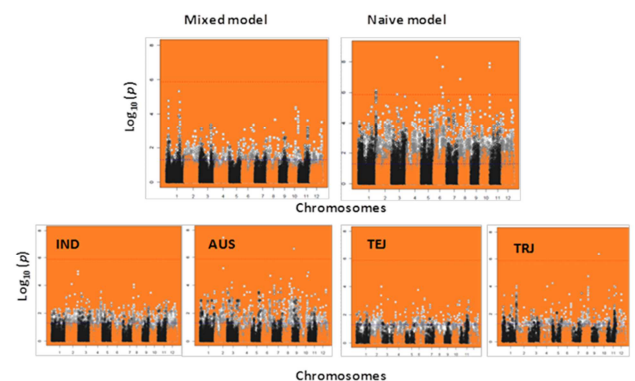


Figure 4. P-values of rice diversity panel from the naïve model, mixed model, and each subpopulation (aus, indica, tropical japonica and temperate japonica) for leaf vein density at eight weeks after sowing.

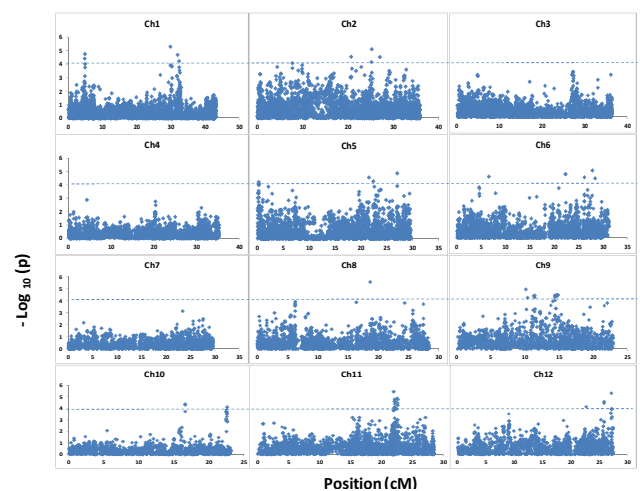


Figure 5. rice diversity panel of leaf vein density. Data corrected for population structure by efficient mixed-model association. Dashed line shows threshold at $-\log_{10}(P\text{-value}) = 4$.

Table 2. The most significant candidate genes, all the candidate genes were tested for expression density (Fluoresce yellow-green Cyanine dye, Cy3) using rice expression profile database (RiceXPro). The Symbol (✓) refers to a positive expression of (Cy3) and the Symbol (x) refers to a lack of expression in leaves.

Location from 5' end (bp)	RGAP number	Express in leaf tissue (Cy3)	Express in all plant tissue (Cy3)	fl cDNA or EST?	Rice Genome Project annotation
4743591	LOC_Os01g09320	200 -12000	✓	fl_cDNA	NADP-dependent malic enzyme, chloroplast precursor, putative, expressed
29734069	LOC_Os01g51700	1000-4200	✓	EST	ras-related protein, putative, expressed
4851430	LOC_Os01g09510	5000-100000	✓	EST	60S acidic ribosomal protein, putative, expressed
4815845	LOC_Os01g09450	1000-10000	✓	EST	OsIAA2 - Auxin-responsive Aux/IAA gene family member, expressed
4717746	LOC_Os01g09280	100-1250	✓	fl_cDNA	myb-related transcription activator, putative, expressed
4672400	LOC_Os01g09246	100-7000	✓	fl_cDNA	glycine-rich protein, putative, expressed
4904501	LOC_Os01g09570	3000-30000	✓	fl_cDNA	6-phosphofructokinase, putative, expressed
16592705	LOC_Os10g31780	1000-150000	✓		oxidoreductase, short chain dehydrogenase/reductase family domain containing protein, expressed

NADP-dependent malic enzyme, chloroplast precursor (LOC_Os01g09320). NADP-malic enzyme belongs to the family of oxidoreductases. In addition, the systematic name of this enzyme class is (S)-malate: NAD⁺ oxidoreductase (decarboxylating) [26, 27]. Most importantly, a study done by [28] confirmed that NADP-malic enzyme involved in C4 photosynthetic pathway in seven rice genotypes during grain filling stage. Furthermore, this gene is considered to be excellent candidate because it is linked to function in pyruvate metabolism and carbon fixation and has expression intensity in leaf tissue of approximately 12000 Cy3.

Ras-related protein, putative, expressed (LOC_Os01g51700). The *rgp1* is a gene that encodes ras-related GTP-binding protein. [29] reported that transgenic tobacco plants expressing *rgp1*, show different morphological characteristics. For example, some R1 progenies of self-pollinating plants developed abnormal leaf phenotypes, such as the abnormal branching of the main vein leading to the formation of twin or even triple leaves [29]. This may nominate this gene as a good candidate gene. The database RiceXPro reported that this gene had expression intensity in leaf tissue just about 4200 Cy3.

60S acidic ribosomal protein (LOC_Os01g09510). The GTPase large subunit GTPase 1 (LSG1) is responsible for the steps through which the 60S ribosome is been matured. An Arabidopsis mutant *dig6* exhibited multiple auxin-related phenotypes such as altered leaf veins. Furthermore, the expression of this gene was found to be high in regions where the auxin can be accumulated and have role in the development of ribosome [30]. The database RiceXPro showed that this gene had expression intensity in leaf tissue reaching nearly 100000 Cy3.

Auxin-responsive Aux/IAA gene family member (LOC_Os01g09450). Gene expression regulates partially the acts of Auxin. Genes regulated by the Auxin can be classified into many gene families, such as the *Aux/IAA*, the *SAUR* and the *GH3* family [31]. In addition, different growth and development stages of plant are mediated by auxin and are transported all through the plant [32]. The *AXR2* gene was cloned by [33] from Arabidopsis plant showing that it is like to *IAA7* gene, which is one of gene family that is responsible for making morphological responses to normal light.

RiceXPro database showed that this gene had an expression intensity in leaf tissue of roughly 10000 Cy3.

Myb-related transcription activator, putative, expressed (LOC_Os01g09280). MYB proteins constitute a large family of transcription factors [34]. [35] confirm the important role of the MYB family in the metabolism and development of plant. Moreover, RiceXPro database suggested this gene had expression intensity in leaf tissue that reach of about 1250 Cy3.

Glycine-rich protein (LOC_Os01g09246). Glycine-rich protein is a group of proteins that responsible for cell wall structure. This group of proteins occurs in several higher plant species and its expression in vascular tissue of rice was reported by [36]. RiceXPro database showed that this gene had expression intensity in leaf tissue that about 7000 Cy3.

6-phosphofructokinase (LOC_Os01g09570). Isozymes of 6-phosphofructokinase are found in the cytosol and plastid compartments and this gene have role for developing leaf tissue [37]. In addition, RiceXPro database revealed that this gene had expression intensity in leaf tissue around 30000 Cy3.

Oxidoreductase, short chain dehydrogenase /reductase family (SDR) LOC_Os10g31780). This gene has very large family consists of at least 140 different enzymes. The SDR family comprise ABA2 [38, 39]. The ABA2 gene, which involved in ABA biosynthesis, encodes OST1 gene. This gene encodes a protein, which controlled by ABA is responsible for stomatal closure [40]. In addition, RiceXPro database showed that this gene had an expression intensity in leaf tissue namely about 150000 Cy3.

Improving high yielding rice genotype is a target of rice breeders. Therefore, understanding the mechanisms affecting leaf vein density is an important matter for rice breeding. However, the mechanisms underlying leaf vein density are complicated.

4. Conclusion

Detecting quantitative trait loci (QTLs) which confer leaf vein density promises to speed up the aim of this experiment. In this study differences in leaf vein density between rice genotypes has been exposed and QTL identified. These data

point out areas of the rice genome containing genes of potential value in breeding leaf vein density rice. These candidate genes are worthy further investigation.

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