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A global analysis of QTLs for expression variations in rice shoot at early seedling stage

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Running head: eQTL analysis in rice shoot

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Summary

Analyses of QTLs for expression levels (eQTLs) of the genes reveal genetic relationship between expression variation and the regulator, thus unlocking the information for identifying the regulatory network. Oligo-nucleotide expression microarrays hybridized with RNA can simultaneously provide data for molecular markers and transcript abundance. In this study, we used Affymetrix GeneChip Rice Genome Array to analyze eQTLs in rice shoots at 72 h after germination from 110 recombinant inbred lines (RILs) derived from a cross between Zhenshan 97 and Minghui 63. Totally 1,632 single feature polymorphisms (SFPs) plus 23 PCR markers were identified and placed into 601 recombinant bins, spanning 1,459 cM in length, which were used as markers to genotype the RILs. We obtained 16,372 expression traits (e-traits) each with at least one eQTL, resulting in 26,051 eQTLs in total, including both *cis*- and *trans*-eQTLs. We also identified 171 eQTL hotspots among rice genome, each of which controls transcript variations of many e-traits. Gene Ontology analysis revealed enrichment of certain functional categories of genes in some of the eQTL hotspots. In particular, eQTLs for e-traits involving DNA metabolic process was significantly enriched in several eQTL hotspots on chromosomes 3, 5 and 10. Several e-traits colocalizing with *cis*-eQTLs showed significant correlations with hundreds of e-traits, indicating possible co-regulation. We also detected correlations between the QTLs for shoot dry weight and eQTLs, revealing possible candidate genes for the trait. These results provided clues for identification and characterization of regulatory network in the whole genome at the transcriptional level.

Introduction

Understanding of the functional genome involves multiple layers. Availability of the genome sequences, which are accumulating rapidly in recent years, is the prerequisite that provides the structural information of the genes and genome. There have also been large efforts in recent years in various organisms collectively termed functional genomics with the long term goal to characterize the function of the complete set of annotated genes. Large mutant libraries have been generated in several plant species toward saturating the genomes with T-DNA insertions to achieve the goal of obtaining at least one insertion for every annotated gene (Jeon *et al.*, 2000; Alonso *et al.*, 2003; Wu *et al.*, 2003; Krishnan *et al.*, 2009). Tremendous efforts have also been invested in the last two decades to identify genes and QTLs using high density molecular marker linkage maps, resulting in hundreds of mapped genes and QTLs in a large number of organisms including many plant species. Map-based cloning has been practiced to isolate mapped genes. In plants, many genes controlling important processes of growth and development as well as agriculturally important traits have been isolated (Xing and Zhang, 2010). These genes have provided windows for viewing how genes and the genome function, and for understanding the causal relationships between genes and phenotypes (or traits).

Wright (1968) proposed a net-like structure for the relationship between genome and characters, such that each character is controlled by a large number of genes and substitution of a single gene would in turn affect an array of characters. Progress in functional analyses of genes supports such a net-like view. Thus, understanding the relation between a gene and a character requires characterization of the regulatory network, which is much more complex than a simple gene to character relationship.

There has been rapid progress in the technologies for investigating pathways of gene regulation, and data generated have started to shed light on the understanding of regulatory network. One of the techniques is the genetic analysis of expression levels measured by transcript abundance of individual genes using high density molecular marker linkage maps. Such analyses determine map positions of the loci regulating the transcript abundance of the genes, which are referred to as eQTLs (Hansen *et al.*, 2008; Kliebenstein, 2009). eQTLs so identified would provide information on the number, relative effects and genomic locations of the loci regulating the expression of the genes. At the whole genome level, this information would collectively provide starting points for characterizing the regulatory network of the gene expression. Microarray-based genome-wide expression profiling can be used as a platform for eQTL analysis. Hybridization of microarrays with RNA samples from individuals in a segregating population provides data for the expression level of individual genes, and the expression polymorphisms in the mapping population can also provide high-throughout markers for map construction that can be used for mapping eQTLs (West *et al.*, 2007; Potokina *et al.*, 2008). eQTL mapping has been well underway in many sequenced model organisms, including yeast (Brem *et al.*, 2002; Brem and Kruglyak, 2005), mouse (Schadt *et al.*, 2003; Ghazalpour *et al.*, 2006; Ghazalpour *et al.*, 2008), human beings (Cheung *et al.*, 2003; Monks *et al.*, 2004; Goring *et al.*, 2007) and *Arabidopsis* (DeCook *et al.*, 2006; Kliebenstein *et al.*, 2006; Keurentjes *et al.*, 2007; West *et al.*, 2007), as well as unsequenced plant species, including wheat (Jordan *et al.*, 2007) and barley (Potokina *et al.*, 2008). Surprisingly, however, there has been no similar study reported in rice.

Rice, a major staple food crop, has been adopted as a model of monocot species in plant science research. In this study, we employed 110 RILs from a cross between two indica (*Oryza sativa* L. ssp. *indica*) varieties Zhenshan 97 and Minghui 63, the parents of Shanyou 63, the most widely cultivated rice hybrid in China. Genetic populations derived from this cross have been intensively studied for genetic and molecular bases of grain yield, heterosis, quality, plant height, heading date, disease resistances, seedling vigor and response to low-N stress (Yu *et al.*, 1997, 2002; Tan *et al.*, 1999, 2000, 2001; Chen *et al.*, 2002, 2003; Cui *et al.*, 2002, 2003; Hua *et al.*, 2002, 2003; Xing *et al.*, 2002; Xu *et al.*, 2004; Lian *et al.*, 2005). Hundreds of QTLs for these traits have been mapped, and genes controlling resistance to bacterial blight and grain yield were cloned using genetic materials derived from this cross (Sun *et al.*, 2004; Xue *et al.*, 2008). In our study, Affymetrix GeneChip Rice Genome Array was hybridized with RNA samples from shoots of the RILs at 72 h after germination, and eQTLs were identified using the high-density genetic map based on single feature polymorphisms (SFPs). The eQTLs detected were associated with the QTLs for phenotypic traits (pQTLs) of seedling vigor obtained previously by aligning eQTL hotspots with pQTLs. These data provided opportunities for resolving the transcriptional regulatory network at the whole genome level, and for identifying and cloning genes controlling variations of quantitative traits.

Results

SFP detection and genetic map construction

Shoots from germinating seeds of 110 RILs derived from a cross between Zhenshan 97 and Minghui 63 at 72 h after germination were collected for RNA isolation, which was hybridized with Affymetrix arrays. A total of the 226 Affymetrix arrays were generated by hybridizing with RNA samples from 110 RILs each with two biological replicates, and the two parents each with three biological replicates. Analysis of the dataset from perfect match (PM) probes using the SFP detection program *SFPdet* based on a median polish method (Tukey, 1977) (Figure S1) identified 3,504 raw SFPs. After filtering and processing, 2,421 SFPs distributed in 1,731 probesets were obtained. Only one SFP per probeset with the least missing data was selected and used in subsequent analyses.

The 1,731 SFP markers were ordered by their physical locations in the rice genome, based on TIGR Rice Genome Pseudomolecules Release 5.0 (TIGR5.0) (Ouyang *et al.*, 2007). There were three big gaps in previous RFLP (restriction fragment length polymorphism) and SSR (simple sequence repeat) genetic map, located on chromosomes 2, 4 and 9, resulting in 15 linkage groups (Xing *et al.*, 2002). Similar big gaps also existed in the distribution of the SFP markers. We thus resorted to PCR markers to reduce the gaps by developing CAPS (cleaved amplified polymorphic sequence) markers based on SNPs (single nucleotide polymorphisms) that were identified by comparing PCR fragments from the two parents. This effort obtained 23 markers in the gap regions (Table S1). These PCR-based markers and SFPs were treated equally in map construction and eQTL mapping.

We removed from the analysis 99 SFP markers that produced recombination rates ≥0.3 with flanking markers, to avoid ones that could not be incorporated into the genetic map. Examination of the data showed that expression values for 61 of the 99 probesets were detected “Present” only in less than one third of the 110 RILs, thus were error-prone in SFP identification. The remaining 38 SFP markers could not be incorporated likely for two reasons. First, both Zhenshan 97 and Minghui 63 are indica whose genomic sequence is remarkably different from the reference japonica genome of Nipponbare, including inversions or transpositions, in addition to Indels (insertions/deletions) or SNPs (Han and Xue, 2003). Second, non-specific probes resulting from genome duplications may produce false or incorrectly located SFPs as demonstrated by Luo et al. (2007). Markers producing double recombinants between adjacent markers were set to missing in the corresponding RILs, except the three markers in the very big gaps, Os.8125.1.S1_x_at8, S0224 and S0226 (Table S2, S4). Consequently genotype data produced by 1,655 markers in the 110 RILs were accepted (Table S2). Assuming that two RILs with identical recombinant genotype in adjacent makers each had a unique recombination point, the assay of the 110 RILs with the 1,655 markers produced a total of 2,742 recombination breakpoints, about 2.0 breakpoints per chromosome per line. We compared the SFP genotypes of the 110 RILs with those assayed by RFLPs/SSRs (Table S3), and they were highly consistent (Figure S2).

These SFP/PCR markers were grouped into 601 marker recombination bins (Table S4), each referring to a set of markers by which a RIL has a unique genotype except the missing data, with an average of 2.75 markers per bin. In case two or more markers were identified in a bin, we selected the marker with least missing data to represent that bin. As a result, the total missing marker data in all 601 bins for all 110 RILs were 1.66%. The genetic linkage map based on the bins was constructed using the R/qtl package function *est.map* with Haldane map function (Broman *et al.*, 2003). The length of the map was 1,459 cM, with an average resolution of 2.43 cM per bin, corresponding to 619 kb (TIGR5.0). The haplotypes and genetic positions of the 601 marker bins for all the 110 RILs are shown in Figure 1. The steps of the SFP detection and the bin map construction are summarized in Table 1. In the genetic map, there are still three big gaps with genetic distance ≥ 20 cM (20.0 cM and 27.1 cM on chr 2, and 22.2 cM on chr 9), located in the regions where RFLP/SSR markers could not link the chromosomes in the previous map.

Distribution of e-traits

Totally 25,965 (45.25%) of the 57,381 probesets were identified as "Present" for at least one third of the 110 RILs using the algorithm of MAS 5.0 (Liu *et al.*, 2002), which were viewed as expression traits (e-traits). To obtain an overview for the distributions of the e-traits, we used the *t*-test like statistic τ as proposed by Bessarabova *et al.* (2010) to distinguish between one-modal (normal) and wider (bi-nomal or multi-mormal) distirbutions. To do so, we simulated normal distribution data for 10,000 populations each containing 110 numbers (μ=0, σ=1), resulting in a threshold τ = 3.22 (*P*=0.01). By this analysis, distributions for 6,320 (24.3%) of the e-traits exhibited bi-nomal distributuons (τ >3.22) of various patterns (Figure S3). The remaining 19,645 (75.7%) e-traits were considerred to be one-model ($\tau \leq 3.22$), of which 14,681 featured normality (*P*<0.01) as analyzed using Shapiro-Wilk normality test (Royston ,1995).

We also tested transgressive segregation using the method described by Brem and Kruglyak (2005). The distributions of expression levels of 8,006 (30.8%) e-traits in the RILs significantly (*P*<0.05, 1,000 permutations) fall outside the ranges of parental values.

Identification of eQTLs

Composite interval mapping (CIM) (Zeng, 1993, 1994) was applied to identify eQTLs of the expression values of the e-traits with a walking speed of 2 cM along the genetic map of the 601 bins. A logarithm of odds (LOD) threshold 3.12, obtained by global permutation test, was applied to declare the eQTLs. If the genetic distance of two adjacent peaks was more than 20 cM, and the depth of the valley of two adjacent peaks exceeded 2 LOD, the two peaks were regarded as two distinct eQTLs for the e-trait. The eQTL support interval of 1.5 LOD drop on each side from the maximum LOD value was estimated and the maximum support interval size was set to 10 cM from each side.

We obtained 29,116 eQTLs at a genome-wide significant level $P \leq 0.05$, and 18,334 (70.65%) of the total 25,965 e-traits had at least one eQTL. Only the probesets each with TIGR5.0 unique locus support were used for subsequent analysis, resulting in 26,051 significant eQTLs for 16,372 e-traits (Table S5). The number of eQTLs for each e-trait varied from 1 to 6. We anchored the genetic map to the physical map of rice genome (TIGR5.0) based on physical/genetic locations of the markers, and plotted the e-traits and their corresponding eQTLs according to their genetic locations (Figure 2).

Cis/*trans* regulators were identified according to the genetic locations of the e-traits and their corresponding eQTLs. A *cis*-eQTL was defined if its e-trait was located in the eQTL support interval within the accepted drop of 1.5 LOD from the peak LOD value, otherwise it was a *trans*-eQTL. However, proper support intervals could not be defined for some of the eQTLs located in the tips of the chromosomes, in which case a *cis* was defined if the distance of an e-trait and its e-QTL was less than 10 cM, or it was *trans* otherwise. Totally 4,464 *cis*-eQTLs were assessed, as shown in the diagonal of Figure 2. The *cis*-eQTLs tended to have higher LOD scores than *trans*-eQTLs as indicated by the color key in Figure 2. Also *cis*-eQTLs in general had greater effects on the e-traits: 63.4% of the *cis*-eQTLs individually explained >20% of the expression variation of the corresponding e-traits, 14.7% explained 40% to 60% and 10.2% explained >80%, while 95.8% *trans*-eQTLs accounted for <20% variation of the e-traits and only 0.23% *trans*-eQTL explained >80% (Figure 3). As expected, an e-trait with a very large effect eQTL (LOD>40) typically shows a bimodal distribution, and most of bimodal e-traits had a *cis*-eQTL with very large effect as evaluated by LOD and variance explained.

Inspection of the estimated genetic effects of the eQILs showed that 3.0% of the eQTLs had an addictive effect ≥ 0.5 (Table S5), corresponding to a 2-fold change (log2(2)=1) between the two homozygotes. And 91.8% of them could individually explain ≥50% variations of the e-traits, the majority of which were *cis*-eQTLs.

eQTL hotspots

It is also clear from Figure 2 that some regions, e.g. regions on chromosomes 3, 5 and 10, had larger numbers of eQTLs as indicated by the thick horizontal lines. The distribution of the eQTLs deviated significantly (χ^2= 6924.86, *P*<2.2e-16) from random occurrence on the 12 chromosomes. The standardized residues for numbers of the eQTLs, each of which followed a normal distribution asymptotically, on chromosomes 3, 5 and 10 were much larger than those of other chromosomes (Table 2). Thus some regions on these chromosomes are enriched for eQTLs, indicating that there may exist major regulators that control expression of sets of genes.

We investigated the distribution of eQTLs along the genome by dividing the whole genome into 1 cM partitions, and the number of eQTLs in each segment was counted. A permutation test was used to assess the statistical significance of deviation of the observed eQTL distribution per cM from the expectation based on chance events assuming a uniform distribution throughout the genome. In the permutation, each eQTL was randomly assigned to a 1 cM interval in the map, and the resulting number of eQTLs in each interval was counted. The results of 1,000 permutations showed that with *P*≤0.01, the cutoff number of eQTLs per cM by chance alone would be 39, and a larger number would be regarded as an e-QTL hotspot. Thus, 171 potential eQTL hotspots were identified by this test (Figure 4).

To evaluate whether there were functional enrichments in the eQTL hotspots, Bioconductor topGO package (Alexa *et al.*, 2006) was used to resolve the e-traits (probesets) whose eQTLs were mapped in the hotspots into different biological processes according to TIGR5.0 Gene Ontology (GO) annotation. Fisher's exact test was applied to assess the statistical significance of over-representation of the 137 Gene ontology biological process categories (level 5) against all the e-traits mapped to the hotspots (Wang *et al.*, 2010). A hotspot was considered to be enriched for a particular GO term if the *P*-value from Fisher's exact test was ≤0.01 and there were at least 5 genes in this GO in the hotspot. Using this criterion, we identified 21 functional terms enriched in 37 of the 171 eQTL hotspots (Table S6).

In particular, we found that DNA metabolism process (GO: 0006259) was significantly enriched in e-traits mapped to five hotspots (chr 3: 401-402 cM; chr 5: 717-718 cM; chr 10: 1205-1206 cM, 1243-1244 cM, 1,244-1,245 cM) (Table S6). Totally 455 e-traits with eQTLs corresponding to 365 TIGR5.0 annotated loci were in the category of DNA metabolism process in our dataset. Two of the three hotspots on chromosome 10, 1243-1244 cM and 1,244-1,245 cM, were adjacent and combined as one 1243-1245 cM. There were one or two *cis*-eQTLs whose corresponding e-traits involving DNA metabolism in each of the three hotspots chr 3: 401-402 cM, chr 5: 717-718 cM, and chr 10: 1243-1,245 cM, suggesting a possible regulatory network for DNA metabolism in each case with the one having the *cis*-eQTL as a putative regulator. But there was no *cis*-eQTL for genes in this functional category in chr 10: 1205-1206 cM, suggesting that the DNA metabolism process mapped to 1205-1206 cM was regulated by genes functioning in other category (ies). We individually calculated correlations of expression levels of DNA metabolism genes between the e-traits having *cis*-eQTLs and the putative target e-traits with *trans*-eQTLs in the three hotspots. The results showed that expression levels of the probesets Os.21451.1.S1_a_at in 401-402 cM, Os.53059.1.S1_x_at and Os.23359.1.S1_at in

717-718 cM, and Os.6722.1.S1_at in 1243-1,245 cM were highly correlated with most of the putative target e-traits with the *trans*-eQTL(s) in the same intervals of the hotspots (Figure S4). It is likely that genes represented by these four probesets may function, among others, as master regulators of the DNA metabolism process.

Expression relationship of potential regulator and the targets

Transcription factors (TFs) and their regulatory targets may provide ideal models for understanding *cis*-acting and *trans*-regulating network. If the polymorphism resides at the locus of a TF, which affects the expression of the TF directly by altering a functional motif in the promoter region or the stability of the mRNA, this TF would be the cause of a *cis*-eQTL. Transcript abundance of its modulating targets involved in various pathways may be affected, causing a cascade reaction of gene expression. Therefore, the TF would be a *trans*-eQTL for the targeted genes.

To investigate the extent of such regulation, we downloaded all the rice TFs from Rice TFDB (http://ricetfdb.bio.unipotsdam.de/v2.1/) (Riano-Pachon *et al.*, 2007). Totally, 1,078 probesets for 939 unique genes were annotated as TFs. We used their expression levels as e-traits to analyze eQTLs, resulting in a total of 1,722 eQTLs, including 235 (13.6 %) *cis*-eQTLs. We calculated correlations of the transcript levels between the 235 TFs and the e-traits with *trans*-eQTLs located in these TF-containing genomic regions (1.5 LOD-drop support intervals). To evaluate the statistical significance of the correlations, a permutation test was conducted by calculating correlations of the transcript levels between two randomly selected sets of 1,000 e-traits producing 1,000,000 correlation coefficients, giving 0.75 or -0.66 as thresholds for significant correlation at *P*=0.01. After removing the TFs that are significantly correlated with less than 5 e-traits, 19 TFs were significantly correlated with 6 to 30 e-traits (Table S7).

We also did similar calculation for the 4,464 genes with *cis*-eQTLs, 99 genes showed significant correlations with 30 or more e-traits that had *trans*-eQTLs located in these gene regions, including 24 genes showing co-expression with 100-425 e-traits (Table S8). These data indicated that genes other than TFs also play important roles as master regulators for gene expression at the whole genome level.

eQTLs and phenotypic QTLs

Variation of gene expression levels constitutes one of the major causes for variations of trait manifestation (Ashikari *et al.*, 2005; Chu *et al.*, 2006; Frary *et al.*, 2000). It is highly likely that many of the eQTLs would be the causes of phenotypic change. We thus assessed relatedness of the eQTLs with phenotypic variations, or phenotypic QTLs (pQTLs) in this population.

Cui et al. (2002) employed the same RIL population to detect pQTLs for seedling-vigor traits. We extracted data of the same 110 RILs for shoot dry weight from that analysis, which was closest to the tissue used in this eQTL study. Plant dry weight was the most important indicator for the status of rice seedling growth and development. Thus analysis of association between phenotypic variation and eQTLs would help dissect the genetic factors controlling early growth characteristics of rice. The genetic map composed of 601 bins was used to identify pQTLs. Three pQTLs were resolved for this trait (Table S9), which is less than 7 detected by Cui et al. (2002). Two of the pQTLs were in common between these two analyses, and the support intervals of the two pQTLs mapped in this study were much narrower (Figure 5), indicating improved mapping precision.

To reveal possible relationship between the pQTLs and eQTLs, their distributions were compared along the whole genome (Figure 5). The support intervals of all three pQTLs *SDW4*, *SDW5-1*, *SDW5-2* for shoot dry weight overlapped with the eQTL hotspots, suggesting the possibility that genes underlying the eQTL hotspots may contribute to the phenotypic variations. To assess the possibility of causal polymorphisms for pQTLs in the segregation population, we also calculated correlations between the phenotype values of the traits and expression levels of e-traits in the regions where support intervals of *cis*-eQTLs and *trans*-eQTLs overlapped pQTLs. Typically the expression levels of several probesets underneath each of the *cis*-eQTLs overlapping pQTLs were significantly (P<0.001) correlated with the phenotypic values of the trait. The absolute values for the majority of the correlation coefficients were 0.3-0.4 (Table S10). Such correlations may be useful for identifying candidate genes contributing to the trait variation. The e-trait with *trans*-eQTLs also displayed significant expression correlation with shoot dry weight. Especially, *SDW5-1*, with larger effect on the trait, had the smaller support interval (6 cM), and 93 e-traits with *trans*-eQTLs in the support interval showed significant correlations (r ranges 0.3-0.5) (Table S10). It suggested that the genes with *cis*-eQTL in the pQTL regions would be associated with early growth characteristics by regulating lots of genes with *trans*-eQTLs. Testing this hypothesis still requires subsequent functional analysis of the likely candidate genes.

Discussion

Molecular markers and the genetic map of the RIL population

Gene expression microarrays hybridized with RNA provide data that can be used simultaneously for developing molecular markers and measuring gene expression abundance in a segregating population. West et al. (2006) described two types of genetic markers from Affymetrix GeneChip expression data to generate haplotypes for 148 RILs in *Arabidopsis*, GEMs (gene expression markers) and SFPs. They used two methods to detect SFPs, parental min-max and RIL distribution (West *et al.*, 2006). It was demonstrated that SFPs developed using the RIL distribution method offer more complete genome coverage than GEMs and greater marker precision than SFPs based on parental min-max method. Potokina et al. (2008) applied a simple algorithm to identify transcript derived markers, including both SFPs and GEMs, from transcript-level variation across 139 double-haploid (DH) lines in barley. Because GEMs are based on extreme allele-specific expression difference, only *cis*-acting GEMs will place a gene at its correct genetic map location, while *trans*-regulated GEMs will map a gene to another genetic location (Luo *et al.*, 2007), which will cause problems if they are used as markers in genetic mapping of QTLs and eQTLs (Wang *et al.*, 2009). In this study, we focused on SFPs based on single probe hybridization intensity, which ignored GEMs. We applied a median polish method to minimize variation due to differential gene expression that impacts the probeset as a whole (Xie *et al.*, 2009), which is similar to *SFPdev* used by West et al. (2006). However, the method (SFP RIL distribution) they used is too stringent and a smaller number of SFP markers would be obtained when applied in our study (data not shown). We applied a more robust version of *K-means* in clustering, and used normally distributed *Z*-scores to define the boundary of the two groups as described by Luo et al. (2007) and Potokina et al. (2008). We used strict criteria to control false discovery rate and distorted allele frequency to avoid poorly reproducible SFPs. It was demonstrated that the method of SFP detection used in this study, a combination of modifications of previously used methods, is more robust and effective in SFP identification.

Using the gene expression microarrays hybridized with RNA in shoot tissue from 110 RILs at 72 h after germination, we detected 1,632 SFP markers and constructed a high-density genetic map with a very low percentage of missing marker data. Compared to previous RFLP/SSR marker map, the bin map constructed with SFPs increased the density from 8.7 cM (Xing *et al.*, 2002) to 2.4 cM between adjacent markers and with higher accuracy. Still we faced difficulty in some genomic regions, and obtained 23 PCR-based markers to fill the gaps which greatly helped the map construction. This is especially the case for the gap on chromosome 2, otherwise this chromosome would still be two disconnected segments. eQTL analysis also revealed very low density of eQTLs located in some big gap regions. This is due to the very low level of DNA polymorphisms between the two parents, Zhenshan 97 and Minghui 63, in these regions, indicating likely common origin of these genomic regions between the two parents in the breeding history.

The results also showed that the length of the SFP bin map constructed in this study is much shorter than RFLP/SSR map reported previously (Xing *et al.*, 2002). An explanation is that there were false double crossovers between adjacent markers that were incorporated in previous map without the knowledge of the DNA sequences that became available only recently. Setting the markers producing double recombinants between adjacent markers to "missing" would improve the quality of the map as true double recombinants would be extremely rare, and the effect should be negligible. We thus concluded that the high-density genetic map based on the SFP markers detected in this study is of high quality.

The implication of the results in regulatory network of transcription

One of the objectives of eQTL analysis is to provide information for possible regulatory network, just like the QTL analyses do for the identification of genes controlling quantitative traits. The results of this analysis provide enormous amount of information for further investigating the regulatory network at the stage covered by the tissue. The eQTL hotspots have been presumed as containing master regulators that are involved in various regulatory pathways (Brem *et al.*, 2002; Keurentjes *et al.*, 2007; West *et al.*, 2007; Potokina *et al.*, 2008). The most obvious objects sought after for finding regulatory network may be the TFs and their downstream targets. Surprisingly, however, the results showed that gene products other than TFs play large roles in regulating gene expression as evaluated by numbers of co-expressing genes (Yvert et al, 2003). Since a number of methods are now available for assaying the interaction of the regulator and its targets, there is no technical difficulty for validating whether the *trans*-eQTLs identified indeed represent a regulatory network, with the e-traits with *cis*-eQTL as the regulator and the e-traits with *trans*-eQTL as the downstream targets.

An interesting feature revealed by the analysis is the enrichment of genes in certain functional categories among the e-traits with *trans*-eQTLs mapped to eQTL hotspots where one or two e-traits with *cis*-eQTL(s) in the same functional class. Further analysis showed that the expression of the *cis*-eQTL was significantly correlated with most of the e-traits. This is exemplified by the analysis of eQTL hotspots which identified the enrichment of the term DNA metabolism. This suggested the likelihood that *cis*-eQTL(s) not only regulates the expression of itself, it may also regulate the expression levels of a large number of e-traits, although the function of this gene as a regulator has yet to be investigated.

Moreover, the eQTL plot indicated that e-traits regulated by *trans*-eQTLs may also have effects on regulation of other e-traits, which in turn may be regulators of still other e-traits. Thus full characterization of the regulatory network should aim at identifying the members involved in various orders of the regulatory hierarchies, and their interplays in the regulatory pathways.

Biological processes and regulatory activities at the germination stage

Germination is the starting point of the life cycle featured by growth of an embryonic plant contained within a seed leading to the formation of the seedling. Germination of seeds is a multifactorial process involving an array of metabolic events (Vázquez *et al.*, 1991). Soon after imbibitions, repair of DNA damaged during maturation drying and rehydration and synthesis of mitochondrial DNA take place. Moreover, DNA synthesis is also associated with post-germinative cell division. New mRNAs are transcribed as germination proceeds, and the majority of these are likely to encode proteins essential for normal cellular metabolism (Bewley, 1997; Vazquez-Ramos and Sanchez, 2003; Howell *et al.*, 2009). GO analysis showed that eQTLs for the e-traits involved in a few particular functional pathways were enriched in some hotspots, notably DNA metabolism process and RNA metabolism process, indicating that these processes are highly active at this stage. Therefore, the results may help unveil the molecular basis of the complex processes in germination and early seedling.

Implication for identifying genes for pQTLs

Natural variation in transcripts provides an important cause of phenotypic variation (Frary *et al.*, 2000; Ashikari *et al.*, 2005; Chu *et al.*, 2006). Associating eQTLs with phenotypic variation or pQTLs may provide an approach for identifying causal genes of important traits thus enhance the understanding of the molecular mechanisms of quantitative traits. Such association can be established by aligning the support intervals of eQTLs with that of pQTLs, and the eQTLs in the pQTL support intervals may contribute to the phenotypic variations. A *cis*-eQTL would reflect the relationship between genotype and phenotype. In *Arabidopsis*, some cloned genes were shown to be *cis*-eQTLs, such as QTLs for flowering time and development (Johanson *et al.*, 2000; Caicedo *et al.*, 2004), confirming that identifying *cis*-eQTLs in pQTL intervals may provide an effective strategy for QTL cloning.

By reanalyzing the data of shoot dry weight available from this same population (Cui *et al.*, 2002), we remapped the QTLs using the high density genetic map constructed in this study. The alignment showed that master regulators may exist in a region where pQTL and eQTL hotspot overlapped that controls hundreds of genes with *trans*-eQTLs involved in various physiological pathways thus causing measurable phenotypic variations. The genes whose expression was significantly correlated with *cis*-eQTL in the support interval of pQTLs for shoot dry weight may provide candidates for the functional genes. However, detailed functional analysis of individual candidate genes is necessary for identification of the genes for the pQTLs as well as the possibility of master regulators.

Experimental procedures

Plant materials

The seeds of 110 RILs and the parents Zhenshan 97 and Minghui 63 were first soaked in water for 48 h in a chamber (Conviron S10H, Controlled Environment Limited, Winnipeg, Canada) set at 25°C/85% relative humidity/dark, and transferred to another chamber (Conviron PVG36, Controlled Environment Limited, Winnipeg, Canada) for pre-germination (35°C, 85% relative humidity, dark). Germination of the seeds was checked every 2 h and germinated seeds were transferred to a growth chamber (Conviron S10H) and incubated for 72 h (25°C, 85% relative humidity, dark). Shoots from 15 seedlings per line were bulk-harvested and frozen in liquid nitrogen for RNA extracting. Two biological replications for each RIL and three for each parent were sampled, one replication at a time.

RNA isolation, purification, labeling, microarray hybridization and scanning were conducted by the CapitalBio Corporation (Beijing, China) according to Affymetrix standard protocols (http://www.affymetrix.com/products/arrays/specific/rice.affx).

Microarray data handling

All the 226 raw Affymetrix CEL files were read into R2.8.1 platform (http://www.R-project.org) by Bioconductor (http://www.bioconductor.org/) package affy (Gautier *et al.*, 2004; Gentleman *et al.*, 2004; R Development Core Team, 2008). For SFP detection, the chip data were subjected to robust multi-array average (RMA) background correction (Irizarry *et al.*, 2003). Then, the 631,066×226 PM probe values were extracted, quantile normalized (Bolstad *et al.*, 2003) and logarithmic transformed. For eQTL analysis, the expression signals of all 57,381 probesets were estimated using the RMA algorithm (Irizarry *et al.*, 2003). We utilized MAS 5.0 to perform the Wilcoxon signed rank-based gene expression presence/absence detection algorithm for all probesets (Liu *et al.*, 2002). Probesets identified as "Present" in at least one third of RIL chip data (110 RILs × 2 replicates) were regarded as expression traits (e-traits) and only these detected as expressed probesets were used in eQTL analysis.

Detection of SFPs

We developed a program *SFPdet* written in R language (can be required from the authors) to detect SFPs between the two genotypes including the following steps:

(1) Every normalized and log2 transformed probeset matrix consisting of 11 PM probes in the 226 chips was subjected to median polish analysis to extract the residuals.

(2) For every probe, 226 residuals were divided into two clusters using the package cluster function *pam*, partitioning (clustering) of the data into *k* clusters "around medoids", a more robust version of *K*-means (Kaufman and Rousseeuw, 1990).

(3) Probes with two non-overlapping clusters were identified. The means (m_1, m_2) and standard deviations (s_1, s_2) of each cluster were calculated. The probability of every member of each cluster belonging to the other cluster was estimated using *Z*-scores $Z_i=(x_i-m_j)/s_j$, where x_i is the score of a member from cluster *i* and m_j and s_j are the mean and standard deviation of cluster *j*. We used a $|Z_i|\geq 2.576$ ($P\leq 0.01$) to obtain 99% probability that one member of a cluster *i* does not belong to the other, smaller Z score was treated as a missing datum. This is similar to the method described by Luo et al. (2007) and Potokina et al. (2008).

(4) A probe was identified as a SFP marker if the clustering of 226 samples meets the following criteria: a) ≤ 3 missing data points in the 226 chips; b) data for ≥ 5 of 6 parental samples were consistently separated into clusters according to their genotypes; c) ≤ 165 (0.75) of 220 RIL samples were grouped to the same cluster. The first two criteria would control false discovery rate and the last criterion control the distorted allele frequency.

SFPs were identified using the *SFPdet* program based on the PM probes. Two replicates of the RILs were genotyped separately with the SFP markers.

For further analyses, data generated by the SFP markers were filtered. Physical positions (TIGR5.0) of the probes in the genome were determined as described by Xie et al. (2009). Probes each with a unique location in the rice genome were used for SFP confirmation. A genotype was assigned to a RIL if the SFP marker had identical genotypes in both biological replicates, or one of the two replicates had a missing datum and the other had an unambiguous genotype, otherwise the RIL was scored as missing. To ensure quality of the markers, SFP markers assigning more than 10% of the RILs as missing were not included in the analysis.

Development of PCR-based markers

To reduce the gaps in the SFP genetic map, PCR-based markers were explored based on SNPs between the two parents. Primers were designed in the big gaps to produce ~500 bp amplified fragments from both parents, which were sequenced to identify SNPs. CAPS markers were developed using the dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html), and used to genotype the RILs by comparing to the parents. If there were no appropriate restriction enzymes for developing CAPS markers, the amplified RIL samples were directly sequenced for assaying the SNPs.

Construction of the genetic map

The selected SFP and PCR-based markers were ordered by their physical locations in the rice genome (TIGR5.0). Related sequences of the RFLPs and SSRs previously mapped in this RIL population (Xing *et al.*, 2002) were obtained from Gramene (http://www.gramene.org), and were located to the rice genome (TIGR5.0) by BLAST analysis (Altschul *et al.*, 1997). Only markers (200 in total) with the physical locations in agreement with their genetic positions were retained (Table S3).

Before constructing the genetic map, all the SFP/PCR markers were grouped and integrated into recombination bins. As some breakpoints could not be determined due to missing data for some markers, we applied the following method to group markers. All markers were ordered by their physical positions in the rice genome (TIGR5.0), if a marker genotype of a RIL was the same as the bin in which the previous marker was assigned to, except the missing data, the marker was defined as a member of the same bin, otherwise it belonged to the next bin. For each bin, we used the marker with least missing data to represent the bin. Genetic positions of the bins were calculated using the R/qtl package function *est.map* with the default Haldane map function (Broman *et al.*, 2003).

eQTL and pQTL mapping

For each e-trait, transcript levels were averaged over the two microarray replicates for each RIL. Composite interval mapping (CIM) (Zeng, 1993, 1994) analysis with a walking speed of 2 cM was employed to analyze each e-trait using the procedure of SRmapqtl and Zmapqtl of QTL cartographer Version 1.17 (Basten *et al.*, 2004). A global permutation threshold (GPT) approach was applied to obtain a genome-wide threshold for statistically significant eQTLs (West *et al.*, 2007; Potokina *et al.*, 2008). A representative null distribution of 100,000 maximum likelihood ratio test (LRT) statistics (100 randomly selected e-traits×1000 permutations) was applied for all 25,965 e-traits. The GPT was computed at 0.05 significant level giving LRT value 14.36, corresponding to LOD value 3.12 as the threshold. For pQTL mapping, the two replicate data for shoot dry weight from Cui et al. (2002) were averaged for CIM.

Accession codes

NCBI Gene Expression Omnibus (GEO): microarray data have been submitted under accession number GSE22564.

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Supporting information

Figure S1. An example of median polish.

Figure S2. Comparison of genotyping of R001 with 1,655 SFP/PCR markers (left) and 200 RFLP/ SSR markers (right).

Figure S3 Fifteen types patterns of bimodal distributions displayed by 24.3% of the e-traits.

Figure S4. Distributions of correlation coefficients in hotspots involving DNA

metabolism process.

Table S1. Information of the 23 PCR-based markers used in this study.

Table S2. Genotypes and map information for all 1,655 SFP/PCR markers for the 110 RILs from Zhenshan 97 × Minghui 63 cross.

Table S3. Genotypes and map information for 200 RFLP/SSR markers in previous study for the 110 RILs.

Table S4. Genotypes and map information for 601 marker bins for the 110 RILs.

Table S5. Details of 26,051 significant gene expression quantitative trait loci (eQTLs) (P<0.05), affecting the expression of 16,372 rice unique probesets.

Table S6. Significant GO terms in each eQTL hotspot.

Table S7. The number of potential target genes for TFs with *cis*-eQTL in its supported interval.

Table S8. The number of potential target genes for e-traits with *cis*-eQTL in its supported interval.

Table S9. The QTLs identified for shoot dry weight.

Table S10. Correlations between phenotype values of shoot dry weight and expression levels of the e-traits located in the 1.5 LOD-drop support intervals in overlapped regions between *cis*/*trans*-eQTLs with pQTLs.

Tables

Table 1. The number of markers obtained in SFP detection and genetic bin map construction obtained in each of the steps.

Process	Markers
Pre-genotyping[a]	3,504
Post-genotyping[b]	2,421
Number of probesets with SFPs[c]	1,731
With addition of PCR-based markers[d]	1,754
Filtering unincorporated markers[e]	1,655
Grouping into bins[f]	601

[a] Running the program *SFPdet* to obtain candidate SFP markers.

[b] Genotyping each RIL according to the genotypes of the two replicates and removing the SFP markers with >10% missing data.

[c] Selecting the SFP with least missing data in a probeset.

[d] 23 PCR-based markers added to fill in the gaps.

[e] Filtering the markers unincorporated into the genetic map and correcting some double crossover genotypes.

[f] Grouping all the markers into bins according to the recombination breakpoints and calculating genetic distance.

Table 2. Statistics of eQTLs on the chromosomes.

Chr.	Total eQTLs	*Cis*-eQTLs	*Trans*-eQTLs	Chromosome length (cM)	Density (eQTLs/cM)	Exp[a]	SR[b]
1	3,392	810	2,582	180.4	18.8	3,221	3.0
2	2,407	391	2,016	169.9	14.2	3,033	-11.4
3	3,724	503	3,221	179.6	20.7	3,206	**9.1**
4	1,446	335	1,111	122.1	11.8	2,180	-15.7
5	4,160	331	3,829	98.5	42.2	1,759	**57.2**
6	1,887	393	1,494	128.5	14.7	2,294	-8.5
7	1,146	262	884	130.3	8.8	2,326	-24.5
8	1,391	299	1,092	99.7	14.0	1,779	-9.2
9	1,466	325	1,141	83.2	17.6	1,484	-0.5
10	2,863	274	2,589	72.4	39.6	1,292	**43.7**
11	1,310	314	996	105.7	12.4	1,887	-13.3
12	859	227	632	89.1	9.6	1,590	-18.3
Total	26,051	4,464	21,587	1,459.3	17.9	26,051	

[a] Expected number of eQTLs based on chromosome size. χ^2=6928.05 (P < 2.2e-16) for the test of goodness-of-fit between the observed and expected numbers of eQTLs on the 12 chromosomes.

[b] SR: standardized residue [=(observed-expected)/√expected], which follows a normal distribution asymptotically. Thus an absolute SR value larger than 2.33 indicates statistical significance at P < 0.01. A positive value indicates that the observed number is greater than expected.

Figure legends

Figure 1. Haplotypes and genetic positions of 601 marker bins for 110 RILs.

The big color box shows the haplotypes, in which each row represents a RIL, and a column corresponds to one of the bins, arranged (left to right) in physical order based on rice TIGR5.0. The 12 rice linkage groups are laid end-to-end and are separated by vertical black lines. The genetic position of each bin in the genome is depicted at the bottom. The length of the genetic map is 1,459 cM. Red boxes, Zhenshan 97 genotype; Blue boxes, Minghui 63 genotype; White boxes, missing genotype.

Figure 2. eQTLs identified using shoot tissue of germinating seeds of RILs from a cross between Zhenshan 97 and Minghui 63.

The x-axis shows the genomic positions of expressed probesets (e-traits), and y-axis indicates the genetic positions of eQTLs. Rice 12 chromosomes are separated by grey lines. The color-key reflects the logarithm of the odds (LOD) scores (LOD scores greater than 100 are set to 100). LOD scores more than 3.12 were adopted as the cutoff point for eQTLs.

Figure 3. Distribution of R^2 values for *cis*-eQTLs and *trans*-eQTLs individually.

(a) The histogram of R^2 values for 4,464 *cis*-eQTLs.

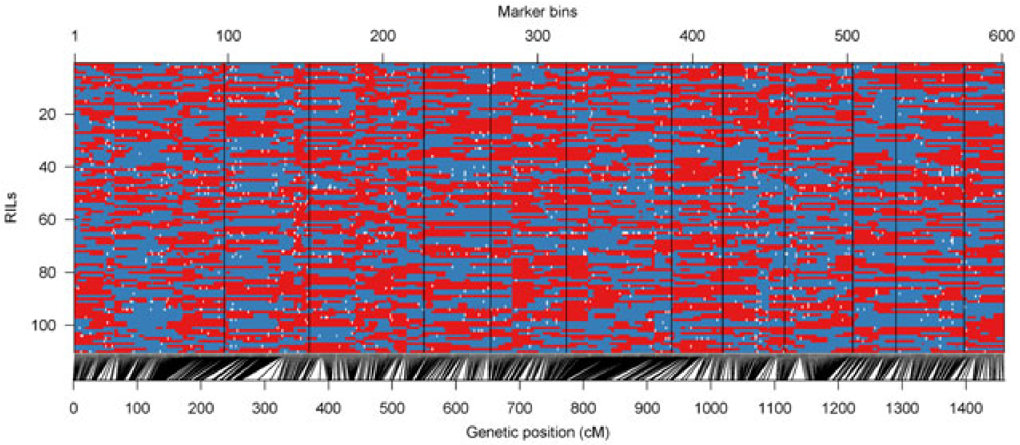
(b) The histogram of R^2 values for 21,587 *trans*-eQTLs.

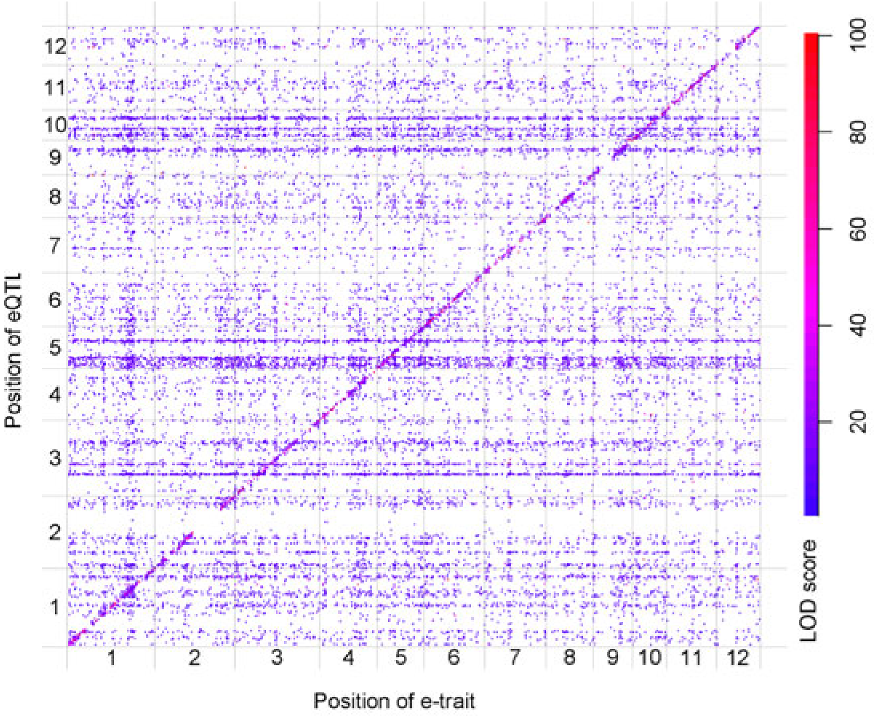
Figure 4. Distribution of eQTLs in the rice genome.

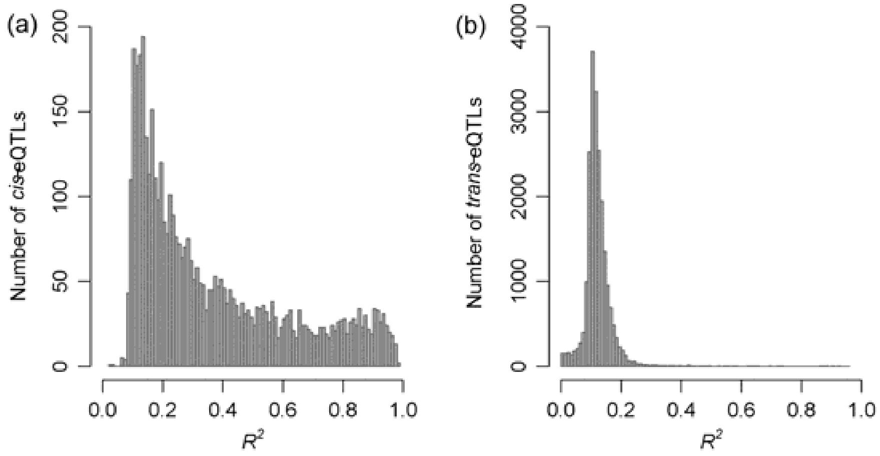
The x-axis indicates genomic locations by chromosomal order. The number of eQTLs (y-axis) is plotted against genome location in intervals of 1 cM. The horizontal line indicates the threshold for eQTL hotspots, represented by the maximum number of eQTLs expected to fall into any interval by chance alone with genome-wide *P*=0.05.

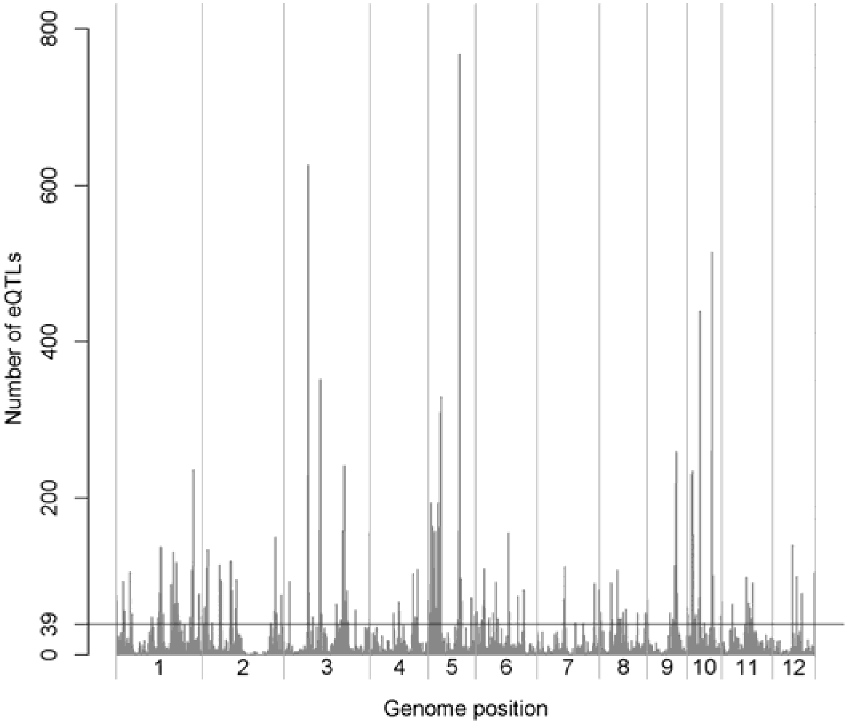
Figure 5. **Comparative distributions of eQTL hotspots and pQTLs for shoot dry weight.**

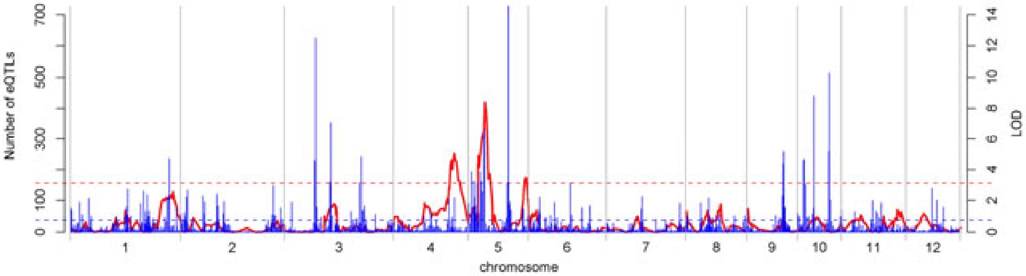
Numbers of eQTLs in intervals of 1 cM are given in blue. LOD statistics of pQTLs for shoot dry weight along the 12 chromosomes are indicated in red. The left y-axis shows the number of eQTLs, with the horizontal blue line showing the global eQTL hotspot threshold 39. The right y-axis shows the LOD scores of QTLs for shoot dry weight, with the horizontal red line indicating the LOD threshold 3.12.


Marker bins
1
100
200
300
400
500
600
RILs
20
40
60
80
100
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
Genetic position (cM)


Position of eQTL
12
11
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
12
Position of e-trait
LOD score
100
80
60
40
20


(a)
200
150
100
50
0
Number of *cis*-eQTLs
0.0 0.2 0.4 0.6 0.8 1.0
R^2
(b)
4000
3000
2000
1000
0
Number of *trans*-eQTLs
0.0 0.2 0.4 0.6 0.8 1.0
R^2


Number of eQTLs
800
600
400
200
39
0
1
2
3
4
5
6
7
8
9
10
11
12
Genome position


Number of eQTLs
0
100
300
500
700
LOD
0
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
9
10
11
12
chromosome