Identification of QTL and candidate genes involved in early seedling growth in rice via high-density genetic mapping and RNA-seq

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ABSTRACT

Early seedling vigor (ESV) is a major breeding target in rice, especially under direct seeding. To identify quantitative trait locus (QTL) affecting ESV, a recombinant inbred line population derived from a cross between 02428 and YZX, two cultivars differing in vigor during early seedling growth, was used for QTL analysis. Nine traits associated with ESV were examined using a high-density map. Of 16 additive loci identified, three were detected in two generations and thus considered stable. Four epistatic interactions were detected, one of which was repeated in two generations. Further analysis of the pyramiding effect of the three stable QTL showed that the phenotypic value could be effectively improved with an increasing number of QTL. These results were combined with results from our previous QTL analysis of the germination index. The lines G58 and G182 combined all the favourable alleles of all three stable QTL for ESV and three QTL for germination speed. These two lines showed rapid germination and strong ESV. A total of 37 candidate differentially expressed genes were obtained from the regions of the three stable QTL by analysis of the dynamic transcriptomic expression profile during the seedling growth period of the two parents. The QTL are targets for ESV breeding and the candidate genes await functional validation. This study provides a theoretical basis and a genetic resource for the breeding of direct-seeded rice.

1. Introduction

Rice (*Oryza sativa* L.) serves as a staple food source for more than half of the global population [1]. With the development of the social economy, rice cultivation and production systems have changed gradually. In recent years, direct seeding of rice has become increasingly popular because of its low cost and simplicity [2]. Direct-seeded rice (DSR) is classified mainly as wet, dry, or water DSR [3]. However, in practice, many factors restrict the development of DSR. For example, in water DSR, rice seeds are completely submerged, leading to poor or no germination and a low emergence rate [4]. In wet or dry DSR, there is no standing layer of water to inhibit the growth of weeds, leading to high weed infestation and, consequently, increased yield loss [5–7]. Seedlings with rapid uniform emergence and high vigor can access nutrients effectively and suppress the growth of weeds [8–10]. In particular, high early seedling vigor (ESV) is crucial for the establishment of seedlings and their eventual biomass production or yield [11]. It is thus desirable to breed rice cultivars with high ESV.

Quantitative trait locus (QTL) mapping is a main tool used to identify genetic factors affecting agronomic traits. Numerous QTL associated with seedling vigor have been detected [11–19]. For example, Zhou et al. [13] used recombinant inbred lines (RILs) and composite interval mapping to identify nine QTL for ESV traits that correlated positively with one another. The individual QTL explained 4%–14% of the total trait variation. Xie et al. [17] mapped eight QTL for ESV using a RIL population and narrowed two major QTL, *qSV-1* and *qSV-5c*, to intervals of 1.13 Mb and 400 kb, respectively. A major QTL for seedling height, *qPHS3-2*, was fine-mapped.

Within the mapped region, the gene *OsGA20ox1*, involved in gibberellin (GA) biosynthesis, was considered the most likely candidate gene [15]. Zhang et al. [18] used a RIL population derived from 93–11 and PA64s and identified 57 QTL for ESV. With a BC₃F₂ population derived from 93–11 and a chromosome segment substitution line (CSSL) harbouring segments from PA64s in the 93–11 background, a major QTL for seedling shoot length, *qSSL1b*, was fine-mapped within 80.5 kb. Recently, He et al. [20] reported that disruption of an isopropylmalate synthase gene, *OsIPMS1*, resulted in low germination speed and seedling growth under various conditions. Further analysis confirmed that regulation of seed vigor by *OsIPMS1* is associated with starch hydrolysis, glycolytic activity, and energy levels in germinating seeds. Although these studies provide us with some valuable information, the data remain insufficient.

High-density single-nucleotide polymorphisms (SNPs) are becoming more readily available owing to the development of sequencing technology [21]. Genetic maps based on high-density SNPs show increased QTL mapping resolution, thus helping improve the efficiency of mapping [22].

In our previous study, we investigated the ESV of 200 conventional rice varieties, among which 02428 has high ESV, and YZX has medium ESV. This indicates that 02428 may carry favourable ESV genes. Therefore, in this study, we used a high-generation RIL population consisting of 192 RILs derived by single-seed descent from the *indica* cultivar YZX and *japonica* cultivar 02428. The genetic map of this population consisted of 2711 bin markers, which were obtained via genotyping-by-sequencing (GBS) [23]. The objectives were, using QTL mapping, to identify further QTL for ESV and, using transcriptome expression profiling, to identify differentially expressed genes as candidates for the QTL. Overall, these results broaden our understanding of the genetic and molecular bases of ESV in rice. In particular, some QTL are strong candidates as promising targets for marker-assisted breeding of DSR varieties. The identified DEGs also provide valuable information for candidate gene verification and the dissection of gene regulatory networks affecting early seedling growth in rice.

2. Materials and methods

2.1. Plant materials

The mapping population contained 192 RILs derived by single-seed descent from a cross between the *indica* YZX and *japonica* 02428 cultivars. RILs from 6th- and 7th-generation populations were planted in respectively the wet and dry seasons of 2016, and the seeds were harvested as experimental materials for each season, and related characteristics were investigated after treatment.

The planting, management, harvesting and storage of the materials followed methods previously reported [24]. The RILs and parents were planted in the experimental field of South China Agricultural University, Guangzhou, China (23.16°N, 113.36°E). Twenty-five-day-old seedlings were transplanted into the experimental field with plant and row spacings both of 20 cm. Plants with the same heading date were selected, and the days to heading of each RIL was estimated; heading date was considered the timepoint when the leading panicle emerged from the leaf sheath to 1 cm. To synchronize seed maturation, six individual plants in the middle of each block were independently harvested on the 35th day after heading in the wet season and on the 40th day after heading in the dry season. The harvested seeds were dried at 42 °C for 5 days and then stored at −20 °C.

2.2. Evaluation of traits related to ESV

Three of the six harvested individual plants were randomly selected, and their seeds were oven treated at 50 °C for 7 days to break dormancy. All seeds were sterilized by soaking in 20% diluted bleach (6%–7% NaOCl) for 30 min and then rinsed six times with sterile water. A total of 100–200 healthy and plump seeds were selected from each plant and placed in a 9-cm-diameter Petri dish onto two layers of filter paper. Then, 10 mL of sterilized distilled water was added, and the dishes were placed in a chamber that had an 8-h light (200 μmol m⁻² s⁻¹)/16-h dark photoperiod at 30 °C. Approximately 2 days later, 10 seeds (whose radicle or germ length had reached approximately 1 mm) per plant were selected, placed in a germination box (length 19 cm, width 13 cm, height 12 cm) and covered with two layers of filter paper, after which 20 mL of sterilized distilled water was added. The culture conditions were as described above. After six days, a WinR-HIZO root image analysis system (Regent Instruments Inc., Québec, Canada) was used to measure the seedling stem diameter (SSD), root length (RL), root surface area (RSA), root volume (RV), and root diameter (RD). Root fresh weight (RFW) and shoot fresh weight (SFW) were measured on a balance, total fresh weight (TFW) was calculated as the sum of RFW and SFW, and seedling height (SH) was measured with a ruler.

2.3. QTL analyses

We used 192 RILs derived from the 02428/YZX cross to map the QTL involved in early rice seeding growth. The RIL population was genotyped by GBS, the Nipponbare genome (IRGSP 1.0) was used as the reference genome for SNP detection, and 85,743 high-quality, biallelic, homozygous SNP markers were obtained. Using a sliding window approach, a high-density linkage map containing a total of 2,711 bin markers was constructed. All bin markers were evenly distributed across 12 chromosomes, the average physical length between the markers was 137.68 kb, and the average genetic distance was 0.86 cM. Among these 12 chromosomes, chromosome 10 had the fewest markers, at 162, and chromosome 3 had the most markers, at 311. The total genetic distance of the genetic map was 2343.68 cM, where Chr. 10 was the shortest chromosome, at 118.78 cM, and Chr. 1 was the longest, at 232.15 cM (Fig. S1) [23]. QTL for ESV were mapped using the BIP function in QTL IciMapping v4.1 software [25]. The inclusive composite interval mapping (ICIM-ADD) method was used to more precisely identify QTL parameters as follows: a 1 cM window size, 500 permutations and a 0.05 type I error rate were employed to call QTL. The logarithm of odds (LOD) score threshold was set to 2.5 [26]. Epistatic QTL were identified via the inclusive composite interval mapping of epistatic QTL (ICIM-EPI) method, with the LOD score threshold set to 5.0 [27]. Genes were annotated and analysed using the Rice Annotation Project (RAP) (http://rapdb.dna.affrc.go.jp) and Ensembl (http://plants.ensembl.org/Oryza_sativa) databases.

2.4. Validation of candidate genes by quantitative real-time PCR (qRT-PCR)

All methods followed our previous report [28]. RNA samples were reverse-transcribed to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA). qRT-PCR was conducted using the AceQ qPCR SYBR Green Master Mix Kit (Vazyme Biotech) according to the standard protocol, and the expression levels of the genes were determined on a StepOne-Plus system (Applied Biosystems, USA). Three replicates were used for each treatment. As an endogenous control, actin was used to normalize the obtained Ct values, and the relative expression values were calculated by the ΔΔCt method. Gene-specific primers

were designed using NCBI primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences of the four candidate genes are listed in Table S1.

2.5. Statistical analysis

Analysis of variance, correlation analyses, t-tests and Duncan's multiple comparisons were performed using Statistical Analysis System (SAS, version 8.01) and Microsoft Excel.

3. Results

3.1. ESV phenotypes of the parents and RIL population

In this study, we investigated the dynamic phenotypic changes in the 9 traits in two parents (Fig. 1). By day 6, the differences in all the traits between the two parents were significant or extremely significant. In particular, SH showed extremely significant differences at all time points; RL and RSA were significant or extremely significant on days 3, 4, 5, and 6; RV was extremely significantly different on days 4, 5, and 6; RFW and TFW were significant or extremely significant on days 2, 4, 5, and 6; and SFW was significant or extremely significant on days 4, 5, and 6. However, RD and SSD became significant or extremely significant only on day 6. In addition, due to lateral root growth, the RD of both materials showed an increase followed by a decrease, while SSD showed a slow increase, and the remaining seven traits showed rapid increases. Notably, on day 6, the SH and RD of YZX were significantly higher than those of 02428, while the remaining 7 traits were higher for 02428 than for YZX. In general, the growth trends between 02428 and YZX were quite different, indicating substantial genetic differences between the two parents, which is beneficial for the identification of QTL.

All nine traits varied widely in the RIL population (Table 1, S2; Fig. S2). In the 6th generation, the coefficient of variation (CV) ranged from 10.25% to 26.44%, and in the 7th generation, it ranged from 10.48% to 28.04%. In particular, the traits showed differing amounts of transgressive segregation. Continuous single-peak distributions were observed for all traits, and the absolute skewness and kurtosis values of each trait were very close to 0, indicating that all of these traits were nearly normally distributed and suggesting that they were controlled by multiple minor-effect genes.

Fig. 2 shows the pairwise phenotypic correlations among the nine traits. The results for the 6th and 7th generations were very similar. There were no strong correlations between aboveground and belowground traits, and the correlation coefficient between SFW and RFW was the largest: 0.62 in the 6th generation and 0.63 in the 7th generation. There was almost no correlation between SH and SSD among the three aboveground traits. The correlation coefficients SFW and SSD and between SFW and SH were similar at approximately 0.60. This result indicated that the contribution rates of SSD and SH to the SFW of early seedling shoots were not very different. Most of the five underground traits showed high pairwise correlations. The correlation between RSA and RL was the highest at 0.93 in both generations. Interestingly, RD showed a negative correlation with RL, with − 0.48 in the 6th and − 0.47 in the 7th generation. This finding was consistent with the change in the root system between 1 and 6 days observed for both parents; that is, with the growth of lateral roots, the diameter gradually decreased (Fig. 1b).

3.2. Identification of additive QTL

QTL analysis revealed 29 QTL (Table 2; Fig. 3). Of the 29, 14 were identified in the 6th and 15 in the 7th generation. The QTL

were distributed on all chromosomes except 5, 7, and 12. The phenotypic variation explained (PVE) ranged from 0.74% to 16.64%. QTL were detected for all nine traits, with the maximum number of QTL (five) associated with SFW and RL and only two QTL each with RD, RFW, SH, and SSD. QTL with the same physical position were considered identical. Of the final total of 16 loci, three were repeatedly detected in the two generations: locus 1 (qRL-1, 6th and 7th), locus 3 (qRSA-3, 6th and 7th; qRV-3, 6th and 7th; qRFW-3, 7th; qTFW-3, 7th), and locus 13 (qSSD-9, 6th and 7th; qSFW-9, 6th; qRFW-9, 6th; qTFW-9, 6th). Locus 9, 12, and 16 were associated with two traits in a single generation. Of the identified loci, four have been reported previously [12,29–31] (Table 2). This finding also confirms that our results are reliable.

3.3. Epistatic interactions

Four epistatic interactions were detected across two generations for three traits (one for SSD, two for SH, and one for RD), distributed on chromosomes 2, 3, 4, 6, 9, and 10 (Table 3). These QTL did not overlap additive QTL. The PVE of these epistatic interactions ranged from 9.25% to 20.00%. One pair of epistatic QTL for SH was detected in both the 6th and 7th generations, and the epistatic effect was negative, indicating that the recombinant phenotypic value was larger than the parental one. In summary, additive × additive epistatic effect is also an important genetic mechanism for ESV in terms of SH, SSD, and RD.

3.4. Identification of the effects of three stable QTL

Among the 16 loci, three, namely locus 1, 3, and 13, were detected in both generations and thus considered stable. To further validate the effects of the three QTL, we first divided the individuals of the RIL population into the 02428 type (marker type 2) and YZX type (marker type 0) for each identified locus and then assessed the differences in the corresponding traits between the two genotypes (Table 4). The mean phenotypic values for all of the corresponding traits of RILs harboring favourable alleles were significantly (P < 0.01) greater than those of RILs harboring unfavorable alleles. Some traits showed QTL in only a single generation, but extremely significant differences were observed between 0-type RILs and 2- type RILs in both generations. This finding suggested that these three stable QTL are credible and could increase the values of the corresponding traits.

To further confirm the three stable QTL with effects on ESV, without considering the effects of interactions among or environmental influences on these QTL. We performed pyramiding analysis of these three QTL, dividing into eight classes the recombinant types of the three QTL. We investigated the distribution of QTL in individual RILs based on the relationship between phenotypic values and the number of favorable QTL alleles present (Fig. 4; Table S3). Although these three QTL did not affect all traits, most of the traits still showed an influence of other additive QTL. All phenotypic values but those of RD increased with increasing aggregation of favorable alleles. In summary, this result indicated that the pyramiding of favourable alleles could improve ESV in the varieties.

3.5. RILs with rapid germination and strong ESV

Both rapid, uniform germination and vigorous seedling growth are important characteristics of DSR cultivars. In our previous studies, we used the same population to identify QTL for seed germination ability at a normal temperature (30 °C). Three QTL, qGI-6, qGI-7, and qGI-9, associated with germination index (GI) with large PVE and stable expression in the 6th and 7th generations were obtained [28]. To identify lines with rapid and uniform seed germination
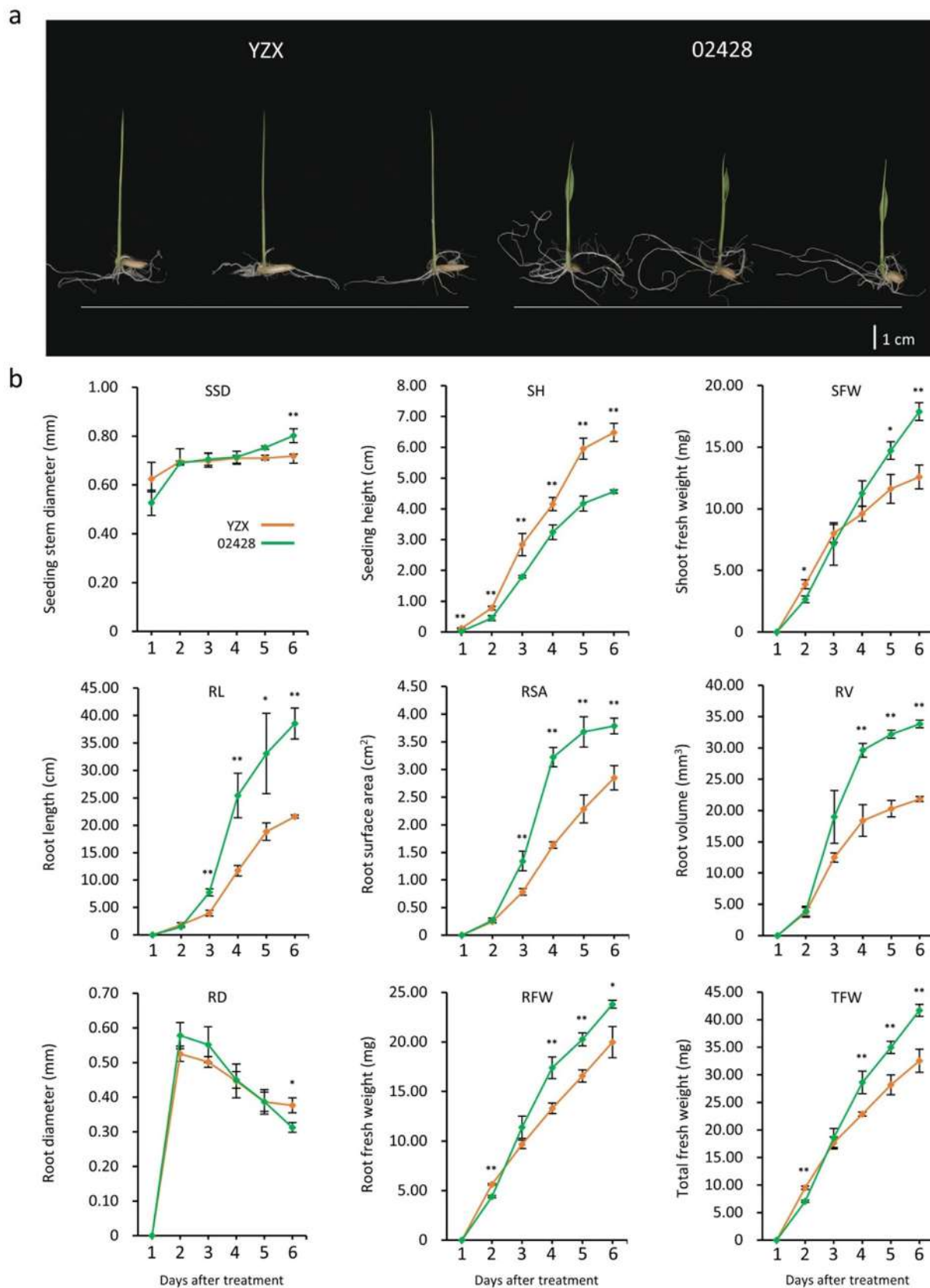
Fig. 1. Phenotypes of 02428 and YZX in the early seedling stage. (a) Phenotypes of both parents after 6 days of treatment; (b) Charts of dynamic changes in nine trait phenotypes.

Table 1
Phenotypic analysis of the YZX, 02428, and YZX × 02428 RIL population.

Trait	Generation	Parents		RIL population				
		02428	YZX	Mean	Range	Skewness	Kurtosis	CV (%)
SSD (mm)	6th	0.90 ± 0.03	0.71 ± 0.04**	0.76	0.60–1.03	0.27	0.12	10.25
	7th	0.80 ± 0.03	0.72 ± 0.01**	0.75	0.57–1.01	0.31	−0.25	10.48
SH (cm)	6th	4.87 ± 0.29	6.58 ± 0.20**	5.44	3.44–8.31	0.28	0.74	13.70
	7th	4.56 ± 0.06	6.48 ± 0.29**	5.35	3.34–8.40	0.43	1.38	14.40
SFW (mg)	6th	21.65 ± 1.01	14.34 ± 0.63**	17.99	11.33–27.76	0.40	0.22	17.44
	7th	17.88 ± 0.72	12.57 ± 0.72**	17.60	9.95–27.91	0.59	0.43	18.56
RL (cm)	6th	39.00 ± 0.44	21.70 ± 0.70**	26.97	6.12–51.13	0.09	1.23	26.44
	7th	38.55 ± 2.80	21.59 ± 0.30**	26.04	4.97–53.03	0.18	1.43	28.04
RSA (cm²)	6th	3.83 ± 0.13	2.77 ± 0.11**	2.58	0.76–4.69	0.04	1.22	24.21
	7th	3.79 ± 0.14	2.85 ± 0.22**	2.48	0.66–4.81	0.09	1.13	25.67
RV (mm³)	6th	32.53 ± 0.48	21.33 ± 1.50*	19.78	6.75–36.58	0.39	0.67	25.98
	7th	33.84 ± 0.60	21.84 ± 0.39**	19.05	6.63–36.75	0.41	0.48	27.44
RD (mm)	6th	0.31 ± 0.01	0.41 ± 0.028**	0.31	0.23–0.44	0.95	1.91	10.55
	7th	0.31 ± 0.01	0.38 ± 0.02*	0.31	0.23–0.41	0.93	1.49	11.01
RFW (mg)	6th	26.21 ± 1.23	21.85 ± 1.24*	19.09	8.13–31.63	−0.08	0.04	22.43
	7th	23.80 ± 0.39	19.98 ± 1.57*	18.44	7.45–33.19	0.21	0.27	24.27
TFW (mg)	6th	47.86 ± 0.53	36.19 ± 1.74**	37.08	19.89–57.28	0.10	0.07	18.06
	7th	41.69 ± 1.11	32.55 ± 2.10**	36.04	17.40–57.30	0.32	0.28	19.42

SSD, shoot stem diameter; SH, shoot height; SFW, shoot fresh weight; RL, root length; RSA, root surface area; RV, root volume; RD, root diameter; RFW, root fresh weight; TFW, total fresh weight.
6th represents the 6th generation; 7th represents the 7th generation. Parents refers to the mean ± standard deviation (SD) of the parents, and * and ** indicate significance at the 0.05 and 0.01 probability levels, respectively.
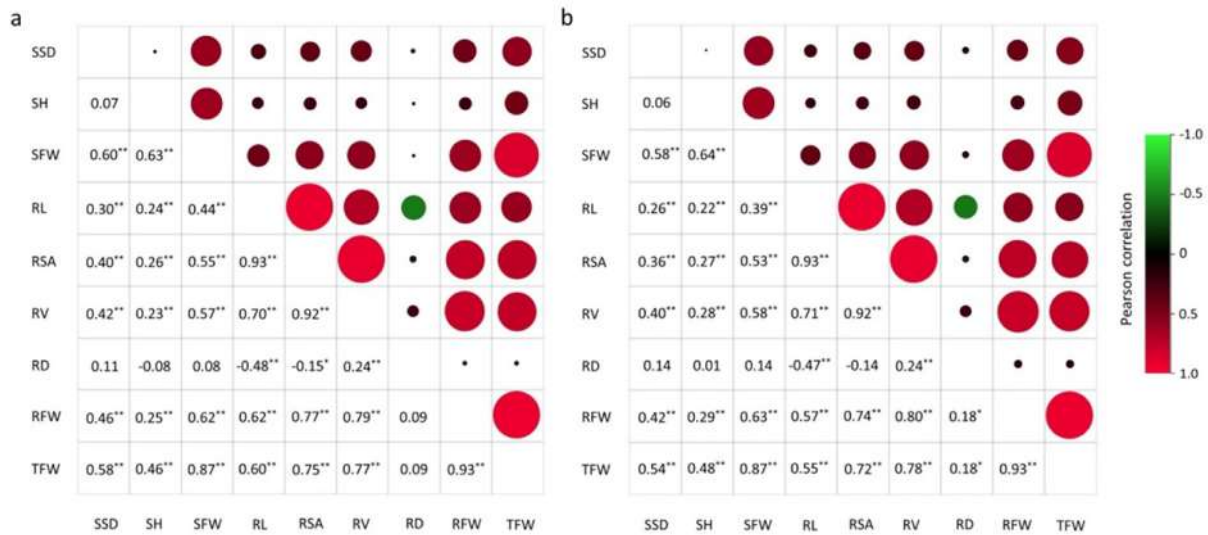CV, coefficient of variation.

Fig. 2. Correlations between nine traits associated with ESV in the RIL population. (a) 6th generation. (b) 7th generation. In the upper diagonal, the size of the circle and the intensity of shading indicate the magnitude of the correlation. The negative correlations are colored green and the positive correlations red. The lower diagonal shows the correlation coefficients, and * and ** represent significance at the 0.05 and 0.01 probability levels, respectively.

and high ESV, we analyzed the genotypes of ESV and GI-associated QTL in this population. According to the source of the favorable allele, pyramiding of the favourable alleles of three QTL for ESV (locus 1, 3, and 13) and three QTL for seed germination speed (*qGI-6*, *qGI-7*, and *qGI-9*) was detected in two RILs (G58 and G182) (Fig. 5a). Taking the phenotypes of the 7th generation as a reference, we found that most values of the 10 traits of G58 and G182, including GI, were higher than the low values of the parents and the mean values of the population, and even transgressive segregation occurred, with a value higher than the high value of the parents. The phenotypes were improved to some extent (Fig. 5b). Thus, pyramiding of the ESV- and GI-associated QTL could increase

the rapid germination of seeds and ESV. Overall, G58 and G182 could serve as favorable allele donors in breeding and increase the efficiency of pyramid breeding of DSR.

3.6. Identification of candidate genes for three stable QTL by gene expression profiling

Markers flanking the QTL were used to anchor them to the reference genome IRGSP-1.0. A total of 259 genes were identified in the QTL regions (Table S4). The list of candidate genes was shortened by analysis of previously completed transcriptomic expression profiles [28]. RNA-seq was performed using RNA extracted from all tissues

Table 2
QTL associated with ESV in two generations.

Locus	QTL	Generation	Chr.	Marker interval	Physical interval (kb)	LOD	PVE (%)	ADD	Known locus
Locus 1	*qRL-1*	6th	1	mk188–mk191	26,250–26,700	2.81	6.02	1.96	
	qRL-1	7th	1	mk188–mk191		3.96	7.93	2.40	
Locus 2	*qRD-2*	6th	2	mk302–mk303	650–750	2.66	6.83	−0.01	
Locus 3	*qRSA-3*	6th	3	mk758–mk763	24,550–25,050	4.27	9.04	0.19	*qSV-3–1*, Zhang et al. [12]
	qRSA-3	7th	3	mk758–mk763		3.68	9.07	0.20	
	qRV-3	6th	3	mk758–mk763		4.69	10.47	1.61	
	qRV-3	7th	3	mk758–mk763		5.02	13.76	1.79	
	qRFW-3	7th	3	mk758–mk763		3.20	8.27	1.29	
	qTFW-3	7th	3	mk758–mk763		3.16	7.78	1.94	
Locus 4	*qSH-3*	7th	3	mk819–mk820	31,150–32,150	3.48	6.98	0.22	Redoña and Mackill [29]
Locus 5	*qRL-4*	7th	4	mk963–mk964	13,450–13,550	2.70	5.41	1.76	Courtois et al. [30]
Locus 6	*qSFW-4*	7th	4	mk974–mk975	14,550–14,650	2.71	0.77	−0.71	
Locus 7	*qSH-4*	7th	4	mk1026–mk1027	21,550–21,650	2.74	5.92	−0.20	
Locus 8	*qRL-6*	6th	6	mk1381–mk1382	6950–7150	3.04	6.57	1.77	
Locus 9	*qTFW-6*	7th	6	mk1403–mk1406	9950–10,300	2.75	6.82	1.75	
	qSFW-6-1	7th	6	mk1403–mk1406		36.90	16.64	3.30	
Locus 10	*qSFW-6-2*	7th	6	mk1415–mk1416	11,600–11,850	28.98	11.72	−2.80	
Locus 11	*qRD-8*	7th	8	mk1915–mk1916	21,200–21,500	2.62	6.55	−0.01	
Locus 12	*qRV-9*	6th	9	mk2124–mk2127	20,850–21,150	3.31	7.44	1.33	Ikeda et al. [31]
	qRSA-9	6th	9	mk2124–mk2127		2.97	6.13	0.15	
Locus 13	*qSSD-9*	6th	9	mk2130–mk2133	21,450–21,850	3.93	9.53	0.02	
	qSSD-9	7th	9	mk2130–mk2133		2.89	6.86	0.83	
	qSFW-9	6th	9	mk2130–mk2133		4.15	11.02	1.36	
	qRFW-9	6th	9	mk2130–mk2133		4.44	10.98	2.19	
	qTFW-9	6th	9	mk2130–mk2133		4.15	10.37	0.03	
Locus 14	*qRL-10*	6th	10	mk2164–mk2165	2550–2650	3.88	8.94	−2.08	
Locus 15	*qSFW-10*	7th	10	mk2235–mk2236	14,550–14,650	2.59	0.74	0.72	
Locus 16	*qRSA-11*	6th	11	mk2505–mk2508	28,150–28,550	3.10	6.52	0.16	
	qRV-11	6th	11	mk2505–mk2508		3.39	7.53	1.32	

Chr., chromosome; LOD, logarithm of odds; PVE (%), phenotypic variation explained (%); ADD, additive effect; a positive value indicates an increasing effect from parent 02428.
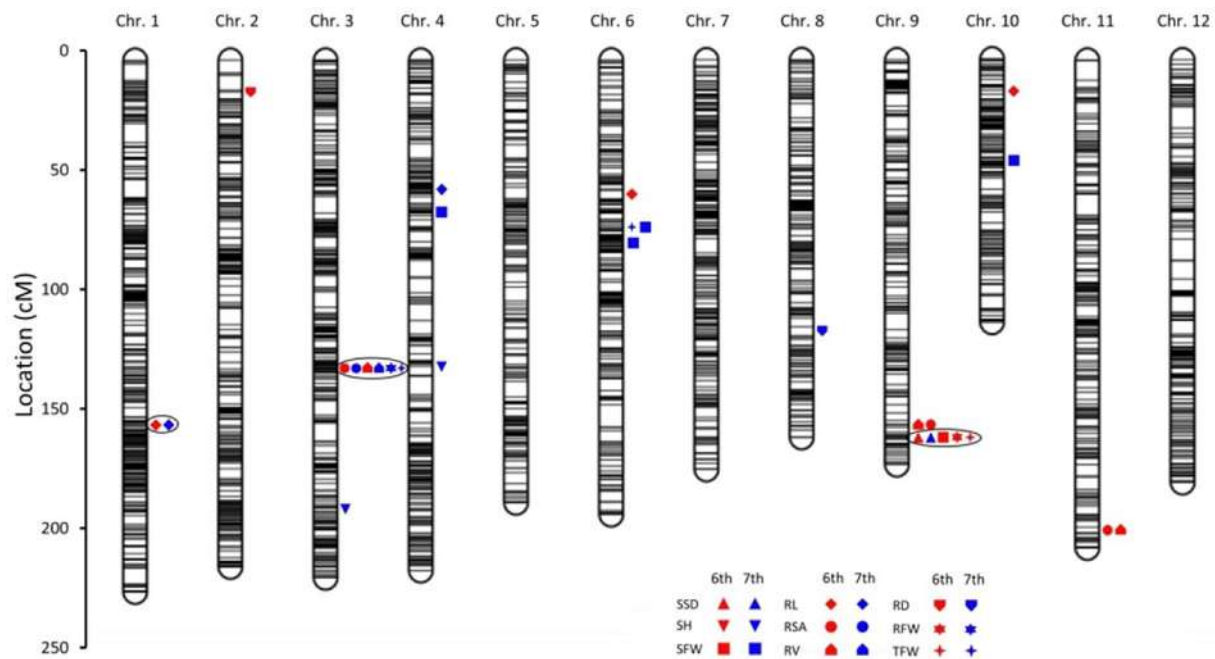
Fig. 3. All QTL positions in the high-density map. The red symbols represent QTL detected in the 6th generation and the blue patterns those detected in the 7th generation. The words associated with different shapes are abbreviations of different phenotypes. The circles represent QTL detected in both the 6th and 7th generations.

(seed + seedling) of germinated YZX and 02428 seeds on days 0, 2, 3, and 4. Considering that the early seedlings of 02428 and YZX all grew rapidly within 1 to 6 days, the difference was obvious. Differential expression profiling (with false discovery rate ≤ 0.05 and absolute value fold change ≥ 2.0) of the two parents was accordingly performed on days 2, 3, and 4, and respectively 5128, 5421,

and 5117 DEGs were detected (Fig. S3). The combination of QTL mapping and expression profiling yielded 34 candidate DEGs for the three stable QTL (Fig. 6; Table S5). Of these DEGs, eight were from locus 1, 11 from locus 3, and 15 from locus 13.

To verify the accuracy of our expression profiling results, we further selected four genes for qRT-PCR according to gene expres-

Table 3
Digenic epistatic QTL in the RIL population identified by interval mapping.

Trait	Generation	Chr. 1	Marker interval 1	Physical interval 1 (kb)	Chr. 2	Marker interval 2	Physical interval 2 (kb)	LOD	PVE (%)	Add1	Add2	AA
SSD	6th	2	mk414–mk415	18,250–18,350	4	mk1006–mk1007	19,550–19,650	5.09	14.42	0.00	0.00	−0.02
SH	6th	3	mk792–mk793	28,400–28,550	10	mk2260–mk2261	17,950–18,050	5.90	15.32	0.06	0.20	0.29
RD	6th	6	mk1437–mk1438	15,150–15,250	6	mk1454–mk1455	17,350–17,450	6.55	9.25	0.03	−0.03	−0.03
SH	6th	9	mk2004–mk2005	6,650–6,750	9	mk2036–mk2037	10,050–10,150	5.57	15.55	0.18	−0.06	−0.33
	7th							6.05	20.00	0.22	−0.10	−0.32

Chr. 1, chromosome ID at the first scanning position; Chr. 2, chromosome ID at the second scanning position.
LOD, LOD score caused by epistatic effects. PVE (%), phenotypic variation explained by epistatic QTL effects (%).
Add1, estimated additive effect of the first QTL; Add2, estimated additive effect of the second QTL; AA, estimated additive by additive effect of QTL at the two scanning positions.

Table 4
Summary of the phenotypic effects of three stable QTL.

Trait	QTL	Generation	Number of RILs of marker type 0	Number of RILs of marker type 2	Donor of positive allele	Phenotypic value		Difference
						Marker type 0	Marker type 2	
Locus 1								
RL (cm)	qRL-1	6th	138	44	2	25.93 ± 6.72	30.11 ± 5.68	4.18**
	qRL-1	7th				24.92 ± 6.97	29.06 ± 6.09	4.14**
Locus 3								
RSA (cm²)	qRSA-3	6th	105	62	2	2.42 ± 0.59	2.79 ± 0.58	0.38**
	qRSA-3	7th				2.32 ± 0.62	2.71 ± 0.62	0.39**
RV (mm³)	qRV-3	6th				18.28 ± 4.54	21.78 ± 5.15	3.49**
	qRV-3	7th				17.49 ± 4.60	21.25 ± 5.40	3.76**
RFW (mg)	–	6th				18.21 ± 4.14	20.22 ± 3.80	2.02**
	qRFW-3	7th				17.31 ± 4.17	19.77 ± 4.29	2.46**
TFW (mg)	–	6th				35.78 ± 6.27	38.86 ± 5.99	3.08**
	qTFW-3	7th				34.33 ± 6.28	38.06 ± 6.55	3.72**
Locus 13								
SSD (mg)	qSSD-9	6th	109	71	2	0.74 ± 0.08	0.79 ± 0.07	0.05**
	qSSD-9	7th				0.73 ± 0.08	0.78 ± 0.07	0.06**
SFW (mg)	qSFW-9	6th				17.44 ± 3.22	18.97 ± 2.87	1.53**
	–	7th				17.02 ± 3.22	18.46 ± 3.21	1.44**
RFW (mg)	qRFW-9	6th				18.16 ± 4.23	20.96 ± 3.76	2.80**
	–	7th				17.60 ± 4.40	20.03 ± 4.31	2.43**
TFW (mg)	qTFW-9	6th				35.60 ± 6.75	39.93 ± 5.88	4.33**
	–	7th				34.62 ± 6.91	38.48 ± 6.70	3.86**

"-" indicates that no QTL was detected during the season.
Difference, phenotype of a favorable allele minus that of an unfavorable allele; ** significant at the 0.01 probability level.

sion level (Fig. 7). They included an *expressed protein* (Os01g0655500), an *OsWAK26-OsWAK receptor-like protein kinase* (Os03g0643250), a *flavin-containing monooxygenase family protein* (Os09g0549300), and an *HMGd1 protein* (Os09g0551600). Transcriptome sequencing of the two materials was not performed on days 5 and 6. Accordingly, qRT-PCR of the four genes was performed on days 4, 5, and 6. On these days, the four genes differed significantly in expression between the two parents, suggesting that they accounted for parental differences in early seedling growth.

4. Discussion

4.1. Appropriate phenotypes can effectively indicate ESV

A rice cultivar with strong ESV trait is desirable for direct seeding [3]. For example, in the water DSR system, seeds are broadcasted into standing water, and rapidly growing buds help make contact with oxygen as soon as possible. A healthy and vigorous root system can help anchor seedlings in soil, thus reducing the population imbalance caused by dead or floating seedlings. In dry and wet DSR systems, one of the major threats is weedy rice [32,33]. Robust seedlings can have a competitive advantage over weeds, and rapid growth of seedlings can allow fields to be irrigated as soon as possible and create a hypoxic environment, inhibiting the growth of weeds. However, the traits previously examined by researchers were not comprehensive enough, especially the indexes of the root system, which were mainly root weight and maximum root length [11,12,18]. The period over which seedling vigor was measured also varied. In the present study, we collected phenotypic data six days after material treatment, which is a suitable irrigation period in production practices. Use of a root scanner to acquire RL, RD, RSA, RV, SSD, and SH values and a balance to measure SFW, RFW, and TFW helped us evaluate ESV comprehensively and improved the efficiency of QTL mapping.

4.2. Utility of combined high-resolution QTL mapping and expression profiling

In general, two parents with large differences in target traits are used to construct populations, facilitating the detection of QTL for the traits [34]. In this study, the mapping population derived from
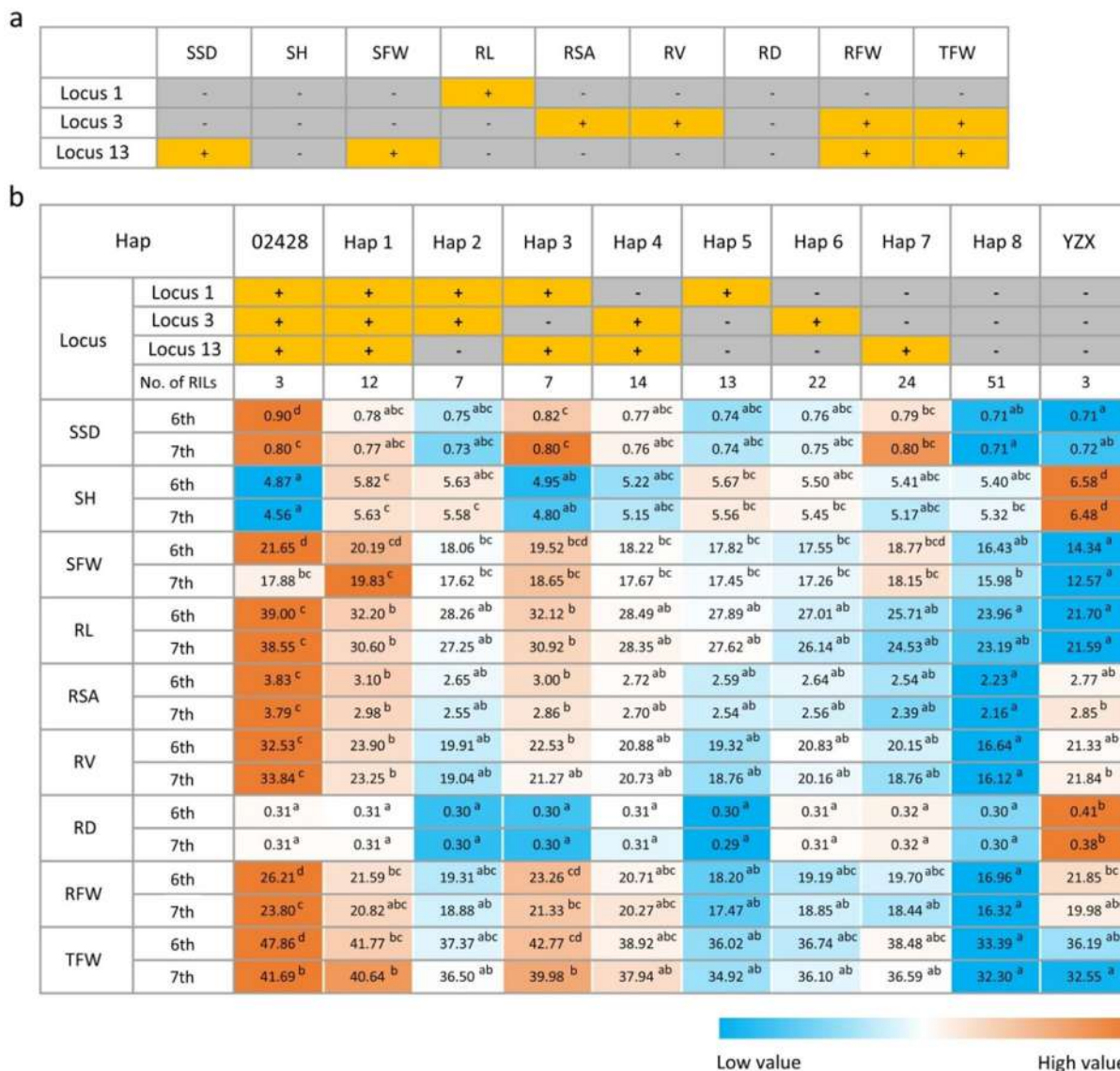
a

	SSD	SH	SFW	RL	RSA	RV	RD	RFW	TFW
Locus 1	-	-	-	+	-	-	-	-	-
Locus 3	-	-	-	-	+	+	-	+	+
Locus 13	+	-	+	-	-	-	-	+	+

b

	Hap	02428	Hap 1	Hap 2	Hap 3	Hap 4	Hap 5	Hap 6	Hap 7	Hap 8	YZX
Locus	Locus 1	+	+	+	+	-	+	-	-	-	-
	Locus 3	+	+	+	-	+	-	+	-	-	-
	Locus 13	+	+	-	+	+	-	-	+	-	-
	No. of RILs	3	12	7	7	14	13	22	24	51	3
SSD	6th	0.90d	0.78abc	0.75abc	0.82c	0.77abc	0.74abc	0.76abc	0.79bc	0.71ab	0.71a
	7th	0.80c	0.77abc	0.73abc	0.80c	0.76abc	0.74abc	0.75abc	0.80bc	0.71a	0.72ab
SH	6th	4.87a	5.82c	5.63abc	4.95ab	5.22abc	5.67bc	5.50abc	5.41abc	5.40abc	6.58d
	7th	4.56a	5.63c	5.58c	4.80ab	5.15abc	5.56bc	5.45bc	5.17abc	5.32bc	6.48d
SFW	6th	21.65d	20.19cd	18.06bc	19.52bcd	18.22bc	17.82bc	17.55bc	18.77bcd	16.43ab	14.34a
	7th	17.88bc	19.83c	17.62bc	18.65bc	17.67bc	17.45bc	17.26bc	18.15bc	15.98b	12.57a
RL	6th	39.00c	32.20b	28.26ab	32.12b	28.49ab	27.89ab	27.01ab	25.71ab	23.96a	21.70a
	7th	38.55c	30.60b	27.25ab	30.92b	28.35ab	27.62ab	26.14ab	24.53ab	23.19ab	21.59a
RSA	6th	3.83c	3.10b	2.65ab	3.00b	2.72ab	2.59ab	2.64ab	2.54ab	2.23a	2.77ab
	7th	3.79c	2.98b	2.55ab	2.86b	2.70ab	2.54ab	2.56ab	2.39ab	2.16a	2.85b
RV	6th	32.53c	23.90b	19.91ab	22.53b	20.88ab	19.32ab	20.83ab	20.15ab	16.64a	21.33ab
	7th	33.84c	23.25b	19.04ab	21.27ab	20.73ab	18.76ab	20.16ab	18.76ab	16.12a	21.84b
RD	6th	0.31a	0.31a	0.30a	0.30a	0.31a	0.30a	0.31a	0.32a	0.30a	0.41b
	7th	0.31a	0.31a	0.30a	0.30a	0.31a	0.29a	0.31a	0.32a	0.30a	0.38b
RFW	6th	26.21d	21.59bc	19.31abc	23.26cd	20.71abc	18.20ab	19.19abc	19.70abc	16.96a	21.85bc
	7th	23.80c	20.82abc	18.88ab	21.33bc	20.27abc	17.47ab	18.85ab	18.44ab	16.32a	19.98abc
TFW	6th	47.86d	41.77bc	37.37abc	42.77cd	38.92abc	36.02ab	36.74abc	38.48abc	33.39a	36.19ab
	7th	41.69b	40.64b	36.50ab	39.98b	37.94ab	34.92ab	36.10ab	36.59ab	32.30a	32.55a

Low value — High value

Fig. 4. Pyramiding analysis of three stable QTL. (a) Summary of the traits associated with the three QTL. A yellow background indicates that the locus is associated with the trait. (b) Pyramiding effects for different numbers of favorable alleles of the QTL. Letters from a to d indicate significantly different values according to statistical analysis using Duncan's multiple range test (α = 0.05).

02428 and YZX showed extreme variation in ESV, and thus was suitable for QTL identification.

In conventional QTL mapping, molecular-marker genotyping is time-consuming and labor-intensive [35]. The markers are mostly distributed at low density and are not able to provide precise and complete information about the numbers and locations of QTL for the corresponding traits [36]. In the present study, we used a high-resolution genetic map containing 2711 bin markers. Compared with previous QTL intervals associated with ESV, the intervals in this study were much narrower and the physical intervals containing the three stable QTL were only approximately 450 kb on average. Another advantage of our study is the integration of QTL mapping and profiling analysis. Numerous studies [16,37–40] have suggested that combining QTL mapping and profiling analysis is a powerful method for successfully reducing the number of candidate genes. We identified 259 genes in the intervals and flanking sequences of the three loci (Table S4), and further combination of these genes with expression profiling data successfully reduced the number of candidate genes to 34.

Finally, we obtained the four most promising candidates. The first candidate is *expressed protein* gene (*Os01g0655500*), whose expression increased with the growth of seedlings in both parents. Moreover, *Os01g0655500* showed significant or extremely significant differences in expression between the two parents at all time points (Table S4; Fig. 7). The second candidate is *OsWAK26-OsWAK receptor-like protein kinase* gene (*Os03g0643250*), which belongs to the wall-associated kinase (*WAK*) gene family, and plays important roles in cell division or growth in *Arabidopsis thaliana* [41]. The third candidate is a *flavin-containing monooxygenase family protein* gene (*Os09g0549300*). *YUCCA* encodes a flavin monooxygenase–like enzyme in *Arabidopsis thaliana*, which catalyses the rate-limiting step in auxin biosynthesis from tryptamine to *N*-hydroxyl tryptamine, and plays an important role in auxin biosynthesis [42]. Therefore, we speculate that *Os03g0643250* and *Os09g0549300* may play a role in the early growth of rice seedlings similar to that observed in *Arabidopsis thaliana*. The last candidate is an *HMGd1* protein gene (*Os09g0551600*). The expression level of *Os09g0551600* in 02428 increased with seedlings growth, which
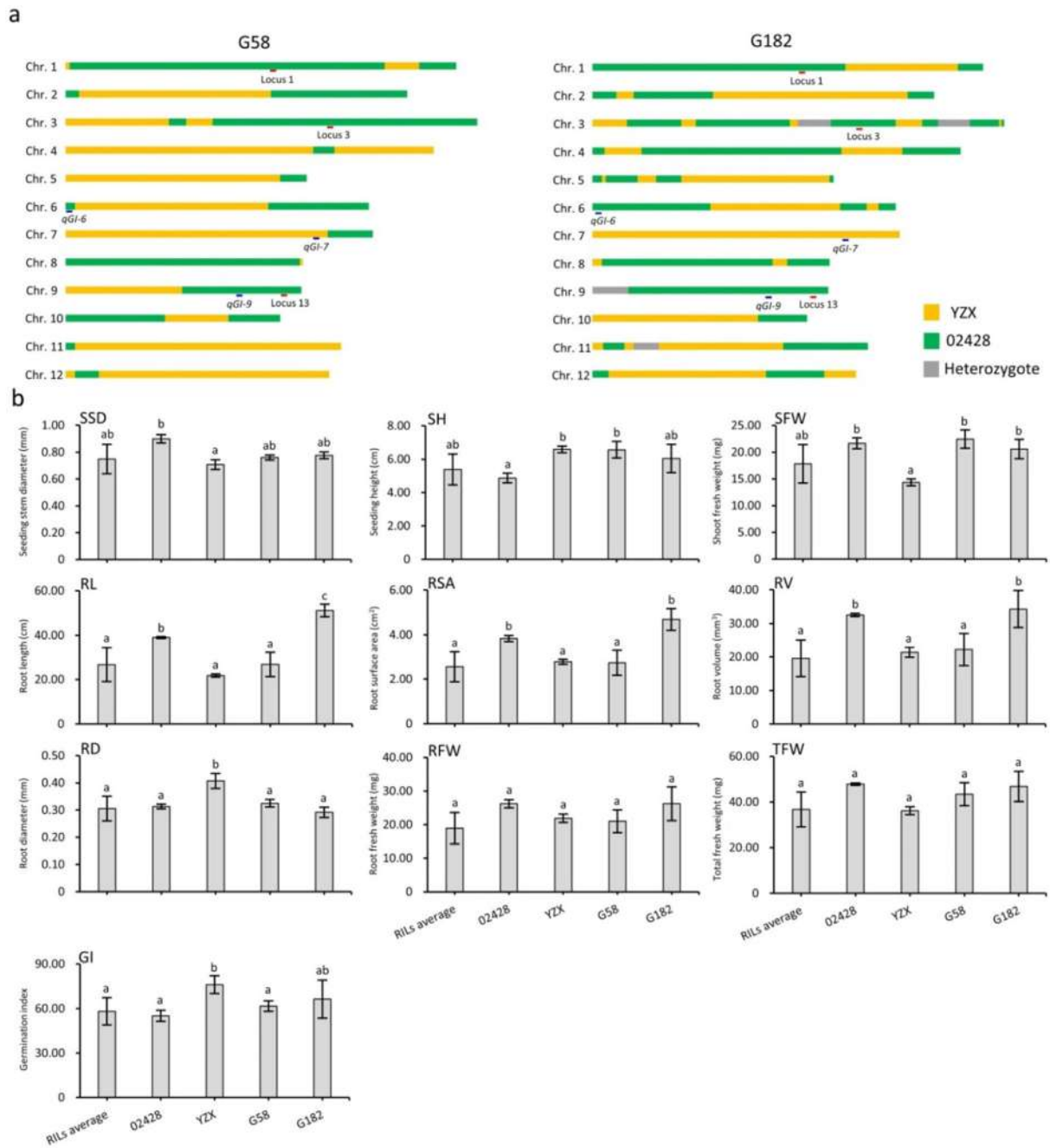
Fig. 5. Pyramiding of loci for ESV and GI in lines G58 and G182 with rapid germination and strong ESV. (a) Genetic background of G58 and G182. (b) Phenotypes of G58, G182, the population mean, and the two parents in the 7th generation.

was consistent with the gene expression atlas of Minghui 63 rice covering the entire life cycle [43]; that is, the gene expression level was high in the seedling stage. However, the expression level of this gene in YZX was almost unchanged (Table S4). These genes provide a foundation for understanding the mechanism of early seedling growth in rice.

4.3. Pyramid breeding is a feasible method for improving ESV

Rice cultivars have been bred specifically for transplanting systems, and ESV has not been selected in conventional breeding for crop improvement programmes [3]. In this study, we identified 16 loci, of which three showed repeatability. Some showed high PVE, for example locus 9 (*qTFW-6*, *qSFW-6–1*, 7th) and 10 (*qSFW-*

6–2, 7th). These loci warrant further examination by fine mapping and candidate gene analysis for ultimate application in marker-assisted selection. Many studies [44–48] have shown that marker-assisted pyramiding of QTL is an effective method for improving traits, particularly quantitative or complex traits. In this study, we tested the pyramiding effect of the three stable QTL in the population by combining favorable alleles.

As expected, aggregating favorable alleles improved the phenotypic traits (Fig. 4). However, aggregation is not the same as simple superposition. We must consider the influence of the environment on genetic locus effects, and epistatic interactions between QTL are also an important factor. In particular, in our study, four epistatic interactions were detected with high PVE (Table 3). Interestingly, our pyramiding analysis, which included two RILs (G58 and
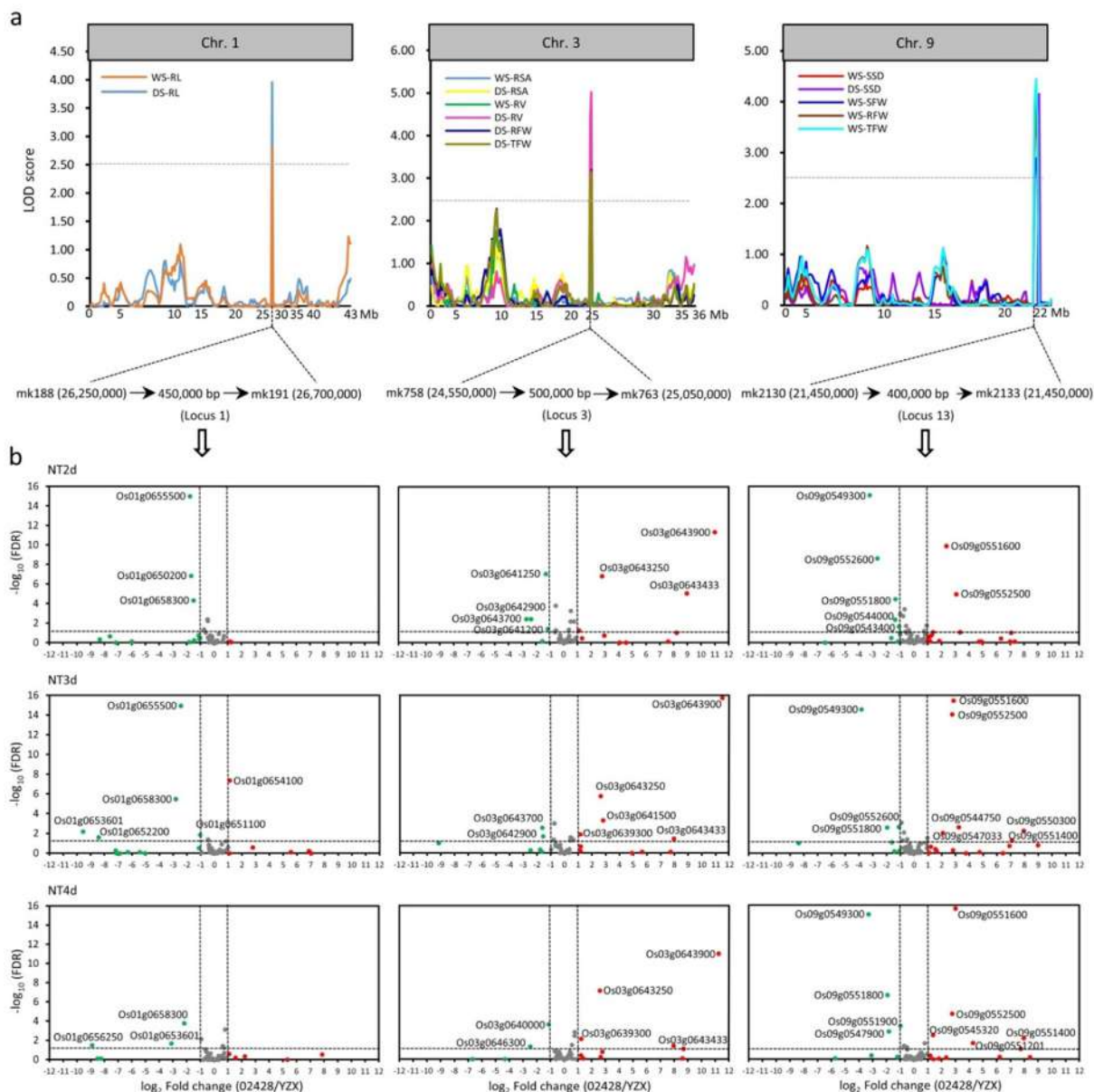
Fig. 6. Candidate gene identification by QTL mapping and expression profiling. (a) Three stable QTL located on chromosomes 1, 3, and 9 identified by QTL analysis. (b) Detection of DEGs by expression profiling on days 2, 3, and 4. Red and green dots represent respectively up- and downregulated genes. Gray dots represent genes with no change in expression.
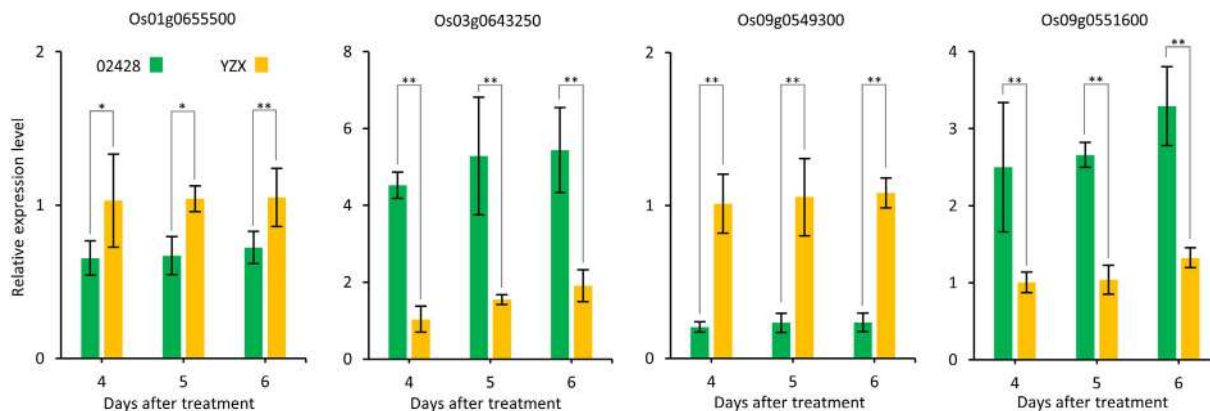

Fig. 7. Relative expression levels of four DEGs. Error bar indicates the standard deviation of mean. * and ** represent significance at the 0.05 and 0.01 probability levels, respectively, according to a *t*-test.

G182) with pyramiding of three rapid-germination QTL alleles, showed phenotypes consistent with our expectations. One of the main reasons may be that the digenic interactions detected in the present study did not involve additive QTL. The actual QTL pyramiding effect awaits further evaluation by marker-assisted selection of QTL in rice accessions with the same genetic background.

CRediT authorship contribution statement

Tao Guo, Zhiqiang Chen, and Jing Yang designed the project, and Jing Yang performed all the experiments andwrote the manuscript. Zhenhua Guo, Lixin Luo, Qiaoli Gao, Wuming Xiao, Jiafeng Wang, and Hui Wangassisted in conducting the experiments and analyzing the data. Zhiqiang Chen and Hui Wang provided thedirection for the study and corrections to the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2020.08.010.

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