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Loci and alleles for submergence responses revealed by GWAS and transcriptional analysis in rice

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Abstract The low seedling rate of rice caused by submergence in the process of rice direct seeding has always been an important factor limiting the popularization of rice direct seeding technology. Improving the tolerance of rice to submergence stress will benefit the production of rice and the promotion of direct seeding technology. In this study, we determined the submergence coleoptile length (SCL), submergence shoot length (SSL), and submergence tolerance index (STI) of 166 different cultivated rice seedlings as the phenotypes. Through the genome-wide association analysis (GWAS) of SCL, SSL, and STI, we found multiple quantitative trait locus (QTL) locations, including nine reported QTL locations. To narrow down the candidate gene numbers, we combined data from GWAS, transcriptomic analysis, gene function annotation, and reported QTL locations, and 50 candidate genes for submergence stress were obtained. Some reported genes had been firstly found to play certain roles in submergence-mediated growth response. Combining with reported RNA-seq data and expression profile data, we focused on four adjacent genes (*LOC_Os11g47550*, *LOC_Os11g47570*, *LOC_Os11g47590*, and *LOC_Os11g47610*) located in *qAG11*. RNA-seq and expression profile suggested the expression of these genes in sensitive and tolerant types differs hundreds of times (146~510 fold). Based on the diverse germplasms, we determined the natural haplotype of these genes. The haplotype analysis of these four genes showed a large genetic difference between *indica* and *japonica*. These results help us to better understand the molecular mechanism of natural variations in submergence tolerance among diverse germplasms and provide materials and new genes for further selection of new submergence tolerance varieties.

Keywords GWAS · Transcriptomic analysis · Submergence · Rice coleoptile · Direct seeding

Hongsheng Gao, Chao Zhang and Huiying He contributed equally to this work.

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Introduction

Submergence and unexpected rain are widespread, recurring problems that negatively affect rice (*Oryza sativa* L.) production in rain-fed lowland areas of South and Southeast Asia (Septiningsih et al. 2009). In these habitats, rice evolved specialized mechanisms to adapt to the environment, such as plant quiescence driven by

the *Sub1A* gene (Fukao et al. 2006; Fukao and Bailey-Serres 2008) and fast plant elongation triggered by *SNORKEL* genes (Hattori et al. 2009).

As a semiaquatic plant, rice could survive under submergence conditions for days. The broad genetic diversity of rice landraces and traditional varieties has enabled its cultivation in different agroclimatic zones and water regimes (Singh et al. 2017). Globally, rain-fed lowlands account for about one-third of the total rice-growing area. Repeated flooding problems in these areas impact rice production. It is estimated that the flood damage caused by flooding replaces the rice yield in rain-fed areas by 20% (Magneschi and Perata 2009). In Asia, since scarcity of labor together with water shortages leads to an increase in farming cost, more and more farmers are opting for direct seeding in both rain-fed and irrigated systems (Miro and Ismail 2013). Enhanced rice submergence tolerance during germination and early seedling growth could improve crop establishment and promote more widespread adoption of direct seeding. In addition, submerging the soil after direct seeding can have a benefit by improving weed control, a major constraint in direct seeding systems (Ismail et al. 2009).

Continuation of carbohydrate metabolism is essential for seed germination and seedling establishment under submergence as previously highlighted in several studies (Miro and Ismail 2013; Ismail et al. 2009; Ella and Setter 1999). Physiologically, due to various metabolic changes occurring under submergence or oxygen-deficiency conditions, plants experience a strong decline in carbon and energy availability (Sasidharan et al. 2018). Enzymes related to starch degradation, glycolysis, and ethanol fermentation, such as a-amylases, phosphofructokinase, fructose-6-phosphate-1- phosphotransferase, alcohol dehydrogenase, and pyruvate dehydrogenase, are highly active under submerged conditions (Kato-Noguchi and Morokuma 2007). As a consequence, the concentrations of sugar and ATP can be maintained to provide energy for survival. In particular, the correlations between submergence tolerance and expressions of *RAMY3D* and *ADH* have been observed by numerous studies (Magneschi et al. 2009).

Among the cloned genes related to submergence tolerance, *Sub1A*, one of the three ethylene-responsive factor (ERF) genes at Sub1 locus, enabled mature rice plants to survive 10~14 days under complete submergence stress (Zhang et al. 2017). *Sub1A* increased the accumulations of the GA signaling repressors Slender Rice-1 (SLR1) and SLR1 Like-1 (SLRL1) and concomitantly diminished GA-inducible gene expressions under submerged conditions (Fukao and Bailey-Serres 2008). *Sub1A* has a positive effect on submergence tolerance of mature rice plant; however, some rice cultivars such as Nipponbare lack this locus but exhibited strong tolerance to submergence during germination (Magneschi and Perata 2009), which suggests a *Sub1A*-independent mechanism may exist during rice germination under submergence (Lee et al. 2009). Ca^{2+} is a signal transducer in plants under anoxic conditions. The calcineurin B-like (CBL) interacting protein kinases (CIPKs) have been found to act as key regulators of energy supply when oxygen availability is restricted (Kurusu et al. 2010). *OsCIPK15*, a CIPK family member, is located on rice chromosome 12. CIPK15 regulates the plant global energy and stress sensor Snf1-related protein kinase 1 (*SnRK1A*) and links O_2-deficiency signals to the SnRK1-dependent sugar-sensing cascade to regulate sugar and energy production and to enable rice growth under submergence (Lee et al. 2009). *OsTPP7* is located in *qAG-9-2*, which was a major quantitative trait locus (QTL) for anaerobic germination tolerance (Angaji et al. 2010). Studies revealed that *OsTPP7* activity increased trehalose-6-phosphate (T6P) turnover, thus enhancing starch mobilization to drive growth kinetics of the germinating embryo and elongating coleoptile. *OsTPP7* finally enhanced anaerobic germination tolerance (Kretzschmar et al. 2015). But in recent report, in the presence of *OsTPP7*, polymorphisms and transcriptional variations of the gene in coleoptile tissue were not related to differences in the final coleoptile length under submergence (Yang et al. 2019). The signal pathway of *OsTPP7* remains unclear at present. The response of rice to anoxic stress is a complex process involving multiple genes and signal transduction pathways, and the related molecular mechanism needs to be further explored.

GWAS has been widely used and becomes a popular strategy to dissect complex traits in rice. GWAS was first used in rice to uncover 14 agronomic traits in landraces (Huang et al. 2010). Submergence tolerance of *indica* and *japonica* rice has been studied by GWAS (Yang et al. 2019). Rice seedlings could survive under water by fast germination and coleoptile elongation (Angaji et al. 2010). At present, there have been studies that used GWAS to explore the difference in flood tolerance between *indica* and *japonica* rice to a certain extent, but there are few phenotypes studied (Zhang et al. 2017; Hsu and Tung 2015; Nghi et al. 2019). Here, we took SCL, SSL, and STI as the submergence phenotype to explore submergence tolerance of rice

seedlings by GWAS analysis and transcriptomic analysis to explore the submergence-tolerant germplasm resources. Combining reported RNA-seq data and expression profile data, we identified the elite natural variation of genes related to submergence resistance. The full excavation of rice planting resources will help to understand the ways and mechanisms of rice's adaptation to related traits, and also help to design and formulate effective breeding strategies to develop related tolerant rice varieties. These results will provide clues to enhance submergence tolerance in breeding program and to explore the genetic mechanism behind.

Materials and methods

Plant materials and growth experiment

A total of 166 rice varieties from 3K project (Table S1) were selected (Wang et al. 2018), and these varieties included three major varietal groups: 89 of *O. sativa indica*, 64 of *O. sativa japonica*, and 13 of *O. sativa Aus*. All the seeds were dried in the oven at 45 °C for 5 days to break dormancy after harvest and then rinsed thoroughly with sterile water after sterilizing in 5% NaClO solution for 30 min. Thirty seeds with uniform germination were seeded in a 96-well PCR plate which has a drainage hole with a diameter of 0.2 cm at the bottom of each well and then submerged in sterile water to a 5-cm depth in a plastic box for the submerging treatment. At the control (normal) treatment, plates floated on the water. The germination experiment was conducted in a growth chamber with a 16 h light (150 μmol m^{-2} s^{-1})/8 h dark cycle at 30 °C for 10 days. The experiment was repeated twice.

Statistical analysis of phenotypic variation

The submergence coleoptile length (SCL), normal coleoptile length (NCL), submergence shoot length (SSL), and normal shoot length (NSL) were measured with five randomly selected seedlings after 10 days. Submergence tolerance index (STI) was calculated as the following formula:

$$STI = SCL/NCL + SSL/NSL$$

Descriptive statistical analysis was performed in Microsoft Office Excel 2010 and R software.

GWAS

Sequence data for all rice varieties used for GWAS were from 3000 rice genome projects (Li et al. 2014). The SNP data were filtered out with minor allele frequencies (MAF) > 0.05 and missing rates < 0.4. EMMAX software was employed for GWAS (Kang et al. 2010). Based on linkage disequilibrium (LD) decay values in *indica* and *japonica* (123 kb and 167 kb, respectively), all genes within less than 100 kb (100 kb upstream and 100 kb downstream of the leading SNPs) of the significant SNPs were considered as candidate genes. Known quantitative trait locus (QTL) was detected by querying the QTL genome viewer Q-TARO (http://qtaro.abr.affrc.go.jp) and Gramene QTL databases (http://archive.gramene.org/qtl/).

RNA-seq data analysis and validation

Based on the phenotype data, four varieties with extreme values (two submergence-sensitive cultivars and two tolerant ones) were selected and then employed for RNA-seq. Total RNA was extracted from coleoptiles of 4 days old seedlings under submergence treatment. RNA samples with 260/280 ratios greater than 1.8 were used. RNA-seq libraries were prepared with two biological replicates for each variety and were sequenced separately using the Illumina Nova sequencer (each sample produced a total size of ~ 6-Gb raw data). The sequencing data generated in this study have been deposited in NCBI's Sequence Read Archive (SRA) database under the series accession number PRJNA624227. As previously described, a false discovery rate (FDR) < 0.05 and absolute value of log2 ratio ≥ 1 were used to identify differentially expressed genes (Benjamini et al. 2001). Cufflinks (version: 2.2.1) software was used to identify differentially expressed gene and transcript expression in RNA-seq experiments (Trapnell et al. 2014). RNA-seq data accuracy verification was based on reported public data (Lasanthi-Kudahettige et al. 2007; Hsu and Tung 2017).

GO enrichment analysis and functional analysis of genes

Gene ontology (GO) enrichment was conducted by AmiGO—online access to ontology and annotation data (http://amigo.geneontology.org/amigo) (Carbon et al. 2009). KEGG pathway analyses of the DEGs were

performed using the public pathway database (https://www.genome.jp/kegg/pathway.html). The gene symbols and functional annotation of genes were based on the public database (http://www.ricedata.cn/index.htm). The physical positions of all genes were based on MSU7.0 annotation and Nipponbare as the reference genome.

Results

Statistical analysis of phenotypic variation with submergence

The results showed that submergence responses of germplasm resources had great variation (Fig. 1a–c). Totally, the *japonica* group responded significantly better than the *indica* group (Table 1). For *indica*, coleoptile and shoot length ranged from 0.73 to 1.97 cm and 3.90 to 16.80 cm, with a mean value of 1.44 cm and 9.19 cm under normal growth condition. While the data ranged from 1.72 to 3.92 cm and 1.20 to 10.60 cm, with a mean value of 2.96 cm and 5.47 cm under submergence. For *japonica*, coleoptile and shoot length ranged from 0.97 to 1.80 cm and 3.70 to 13.40 cm, with a mean value of 1.38 cm and 7.92 cm under normal growth condition, while the data ranged from 1.95 to 4.42 cm and 0.94 to 12.06 cm, with a mean value of 3.36 cm and 5.70 cm under submergence. The coleoptile elongation of *japonica* rice was significantly longer than that of *indica* rice under submergence treatment (Fig. 1d). At the same time, shoot length of *japonica* was also better than that of *indica* (Fig. 1e). These results lead to a larger range and higher mean of STI of *japonica* than *indica* (Fig. 1f). The analysis results of shoot length showed that under submergence treatment plants with longer coleoptile length showed less inhibited shoot growth and higher STI.

GWAS results compared with reported QTLs/genes

To verify the accuracy of the results, significant SNPs in this study were compared with previously detected QTLs using linkage or association mapping approach. The

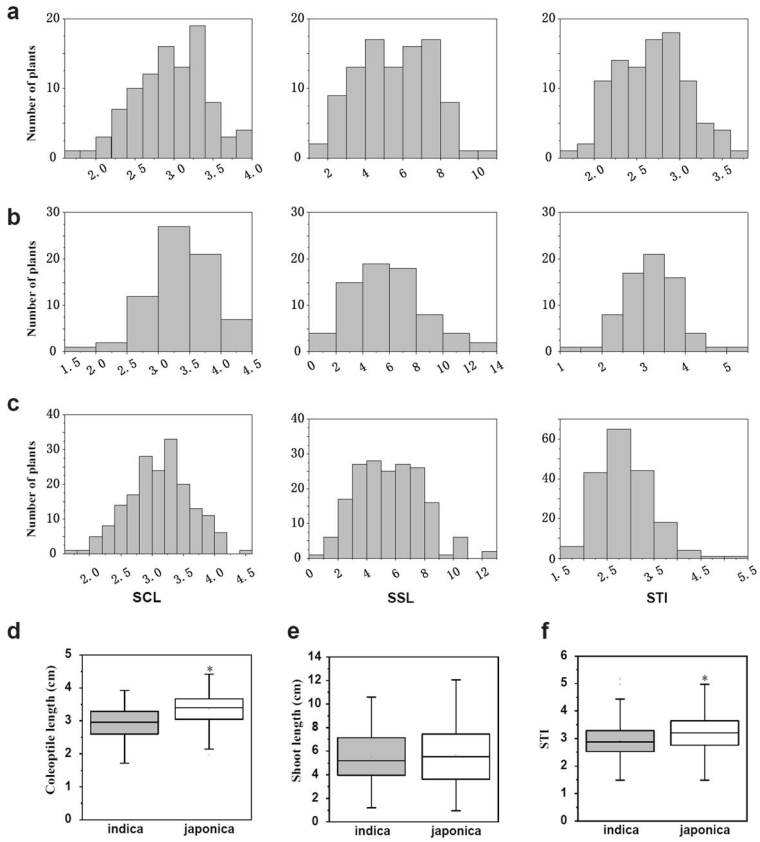


Fig. 1 Phenotypic diversity of submergence responses of different accessions and statistical analysis of phenotypic variation. Phenotypic distribution of **a** *indica* groups, **b** *japonica* groups, and **c** global groups. **d** Box plot of coleoptile length after 10 days of submergence treatment among *indica* and *japonica* groups. **e** Shoot length. f Submergence tolerance index (STI). Data from two replicates. Student's *t* test, asterisk indicates $P < 0.05$

Table 1 Statistical analysis of rice coleoptile and shoot length

Traits	Mean (cm) ± SE	Range (cm)	CV
Indica SCL	2.96 ± 0.05	1.72~3.92	0.16
Indica NCL	1.44 ± 0.02	0.73~1.97	0.14
Indica SSL	5.47 ± 0.21	1.20~10.60	0.37
Indica NSL	9.19 ± 0.25	3.90~16.80	0.26
Indica STI	2.69 ± 0.05	1.74~3.75	0.16
Japonica SCL	3.36 ± 0.06	1.95~4.42	0.14
Japonica NCL	1.38 ± 0.03	0.97~1.80	0.14
Japonica SSL	5.70 ± 0.33	0.94~12.06	0.47
Japonica NSL	7.92 ± 0.24	3.70~13.40	0.24
Japonica STI	3.21 ± 0.09	1.49~5.17	0.22

CV coefficient of variation

results also were compared with previously reported QTL intervals controlling survival rate under submergence. Nine genomic regions were co-localized. In the GWAS of *indica* (Fig. 2a), five loci *qAG1-2*, *qAG3*, *qAG5*, *qAG7.2*, and *qAG11* were detected on chromosomes 1, 3, 5, 7, and 11, where multiple reported QTLs clustered (Angaji et al. 2010; Jiang et al. 2004; Septiningsih et al. 2013; Baltazar et al. 2014; Hsu and Tung 2015). The QTL *qAG1-2*, *qAG3*, and *qAG5* were detected both in SCL, SSL, and STI. In the GWAS of *japonica* (Fig. 2b), seven loci *qAG1-2*, *qAG2.1*, *qAG3*, *qAG6*, *qAG-7-2*, *qAG11*, and *qAG12* were detected on chromosomes 1, 2, 3, 6, 7, 11, and 12. The QTL *qAG3*, *qAG6*, *qAG-7-2*, and *qAG11* were detected both in SCL, SSL, and STI. In the association analysis using all of 166 rice varieties (Fig. 2c), five loci *qAG3*, *qAG5*, *qAG7.2*, *qAG-7-2*, and *qAG11* were detected on chromosomes 3, 5, 7, and 11. The QTL *qAG-7-2* was detected both in SCL, SSL, and STI. It is worth noting that the QTL *qAG3*, *qAG-7-2*, and *qAG11* were detected in at least two different groups (Table S2).

In addition to known QTLs, we have detected some SNPs close to known genes. For example, three adjacent SNPs (chr8_22710591, chr8_22710600, and chr8_23828533) were significantly associated with SCL and STI in the *japonica* and global groups, and these SNPs are close to *RAMY3D* at a distance of 480 kb. The major function of *RAMY3D* is to promote starch amylolysis under energy deficiency (Ismail et al. 2009; Setter et al. 1994; Gibbs et al. 2000; Nagai et al. 2010). *OsTPP7* was reported to be involved in rice anoxia tolerance. In tolerant types, *OsTPP7* accelerates coleoptile elongation in oxygen-deficient environment (Kretzschmar et al. 2015). Here, we identified two

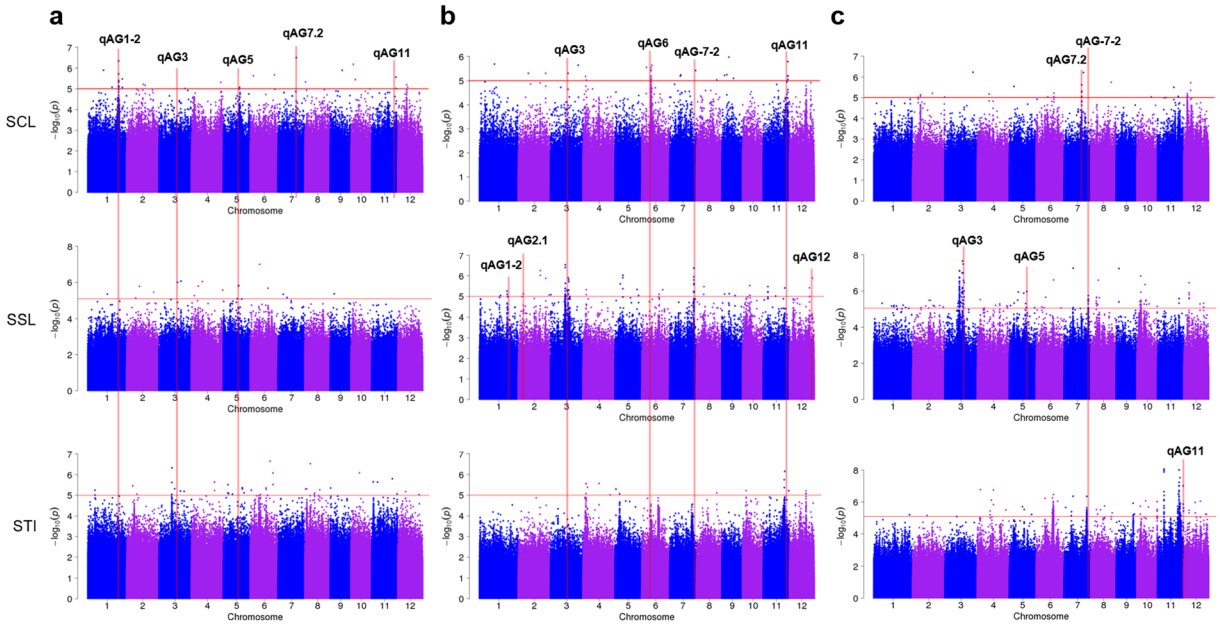


Fig. 2 Manhattan plots for submergence coleoptile length (SCL), submergence shoot length (SSL), and submergence tolerance index (STI). **a** The *indica* group. **b** The *japonica* group. **c** The global. The red horizontal line indicates the significant threshold (−lg(*P*) = 5). Present quantitative trait loci (QTL) were detected by querying the QTL genome viewer Q-TARO (http://qtaro.abr.affrc.go.jp) and Gramene QTL databases (http://archive.gramene.org/qtl/)

significant SNPs (chr9_13225324, chr9_12815761) close to *OsTPP7* at a distance of 560 kb and 970 kb respectively. The other two adjacent SNPs (chr9_5464963, chr9_7623540) were significant in the *japonica* group, and the locus *Sub1* was located between these two SNPs.

RNA-seq data validation and differentially expressed genes response to submergence stress

Based on the phenotype data of SCL, SSL, and STI, we selected four varieties with extreme phenotypes for further RNA-seq analysis (Fig. 3a), including two sensitive varieties (XC190, XC214) and two tolerant varieties (XC93, XC105). The coleoptile lengths, shoot length, and emergence rate of these four varieties were measured after 5 days and 10 days of submergence treatment (Fig. 3b–e).

A total of 2160 differentially expressed genes (DEGs) were identified between sensitive varieties and tolerant ones based on FPKM value (Fig. 4a, Table S3). In order to verify the accuracy of RNA-seq, we compared the results of 2160 DEGs with the public data of previous studies. A total of 384 overlap genes were found in Lasanthi's report, and 324 of them (84.4%) showed the same trend (Lasanthi-Kudahettige et al. 2007). A total of 139 overlap genes were found in Hsu's report, and 95 genes (68.3%) showed the same trend (Hsu and Tung 2015). These results indicated that our RNA-seq data

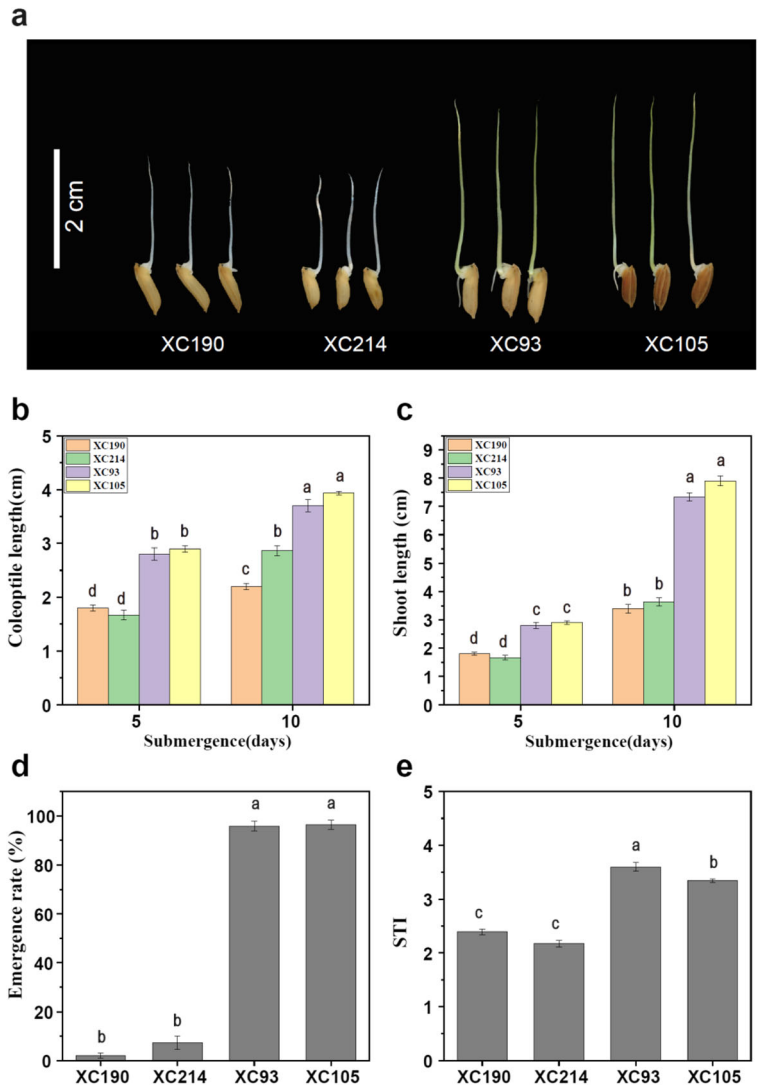


Fig. 3 Phenotypies of XC190, XC214, XC93, and XC105 after 5 days of submergence treatment. **a** The coleoptile length. **b** The coleoptile length after 5 days and 10 days of submergence treatment. **c** The shoot length. **d** The emergence rate after 10 days of submergence treatment. **e** The STI at days 10. Data are shown as mean ± SE from three replicates. Student's *t* test, $P < 0.05$. Scale bar = 2 cm

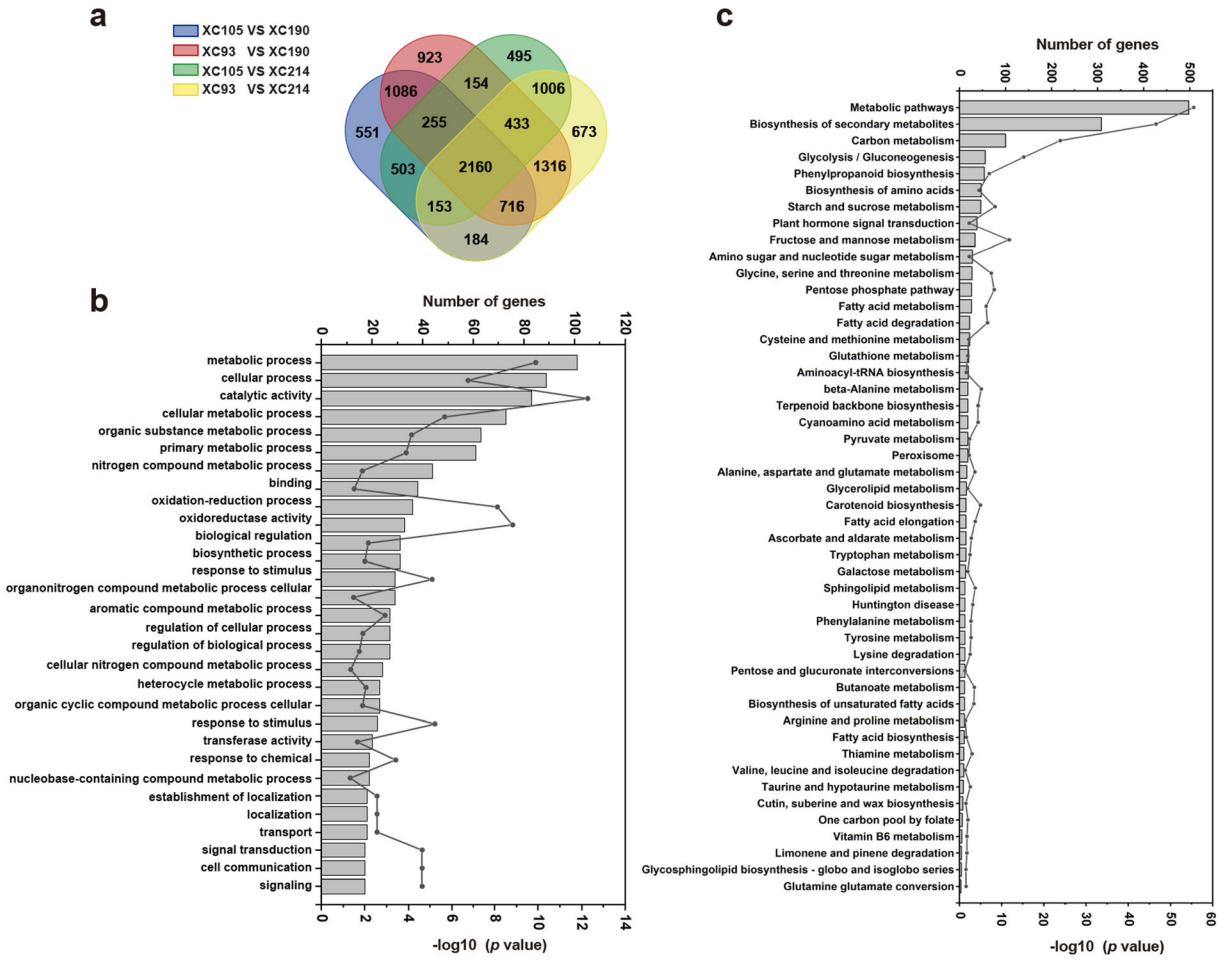


Fig. 4 Transcriptomic analysis of submergence response between tolerant and sensitive rice varieties. **a** Modified Venn diagrams showing the common and specific genes in response to submergence across tolerant and sensitive rice varieties. **b** GO enrichment analysis of DEGs in response to submergence. **c** KEGG pathway analysis of DEGs in response to submergence. All DEGs were selected by a rigorous comparison at adjusted *P* values (FDR) of < 0.05. GO result was simplified, only the top 30 terms by number were exhibited

was credible. GO and KEGG showed that most of these DEGs were enriched in metabolic process (Fig. 4b–c). In addition, the GO results also showed that genes responsible for catalytic activity and transporter activity were specifically enriched in the upregulated gene set in tolerant types, while those responsible for mitochondrial electron transport were downregulated.

Inhibition of energy supply by anoxia is the primary reason for the inhibition of coleoptile elongation by flooding. Due to anoxia, most oxygen metabolism will be replaced by other metabolism to provide energy for rice growth (Miro and Ismail 2013). Here, GO and KEGG also proved this. Many DEGs were enriched in metabolic process and glycolysis pathway. Combining the results of GO and previous reports, we analyzed some important genes involving in glycolysis (Fig. 5, Table S4). Most genes involved in key enzymes of glycolysis were upregulated in tolerant varieties. Such as, alpha-amylase (*LOC_Os08g36910*, *RAMY3D*), Hexokinase (*LOC_Os01g53930*, *OsHXK6*), pyrophosphate-dependent phosphofructokinase (*LOC_Os08g25720*, *PPi-PFK*), phosphofructokinase (*LOC_Os05g10650*, *PFK*), fructose-1,6-bisphosphate aldolase (*LOC_Os06g40640*, *OsAld-Y*), and glyceraldehyde-3-phosphate dehydrogenase (*LOC_Os02g38920* and *LOC_Os08g34210*, *G3PDH*) were significantly upregulated. Meanwhile, the tolerant genotypes showed enhanced expression of alcohol

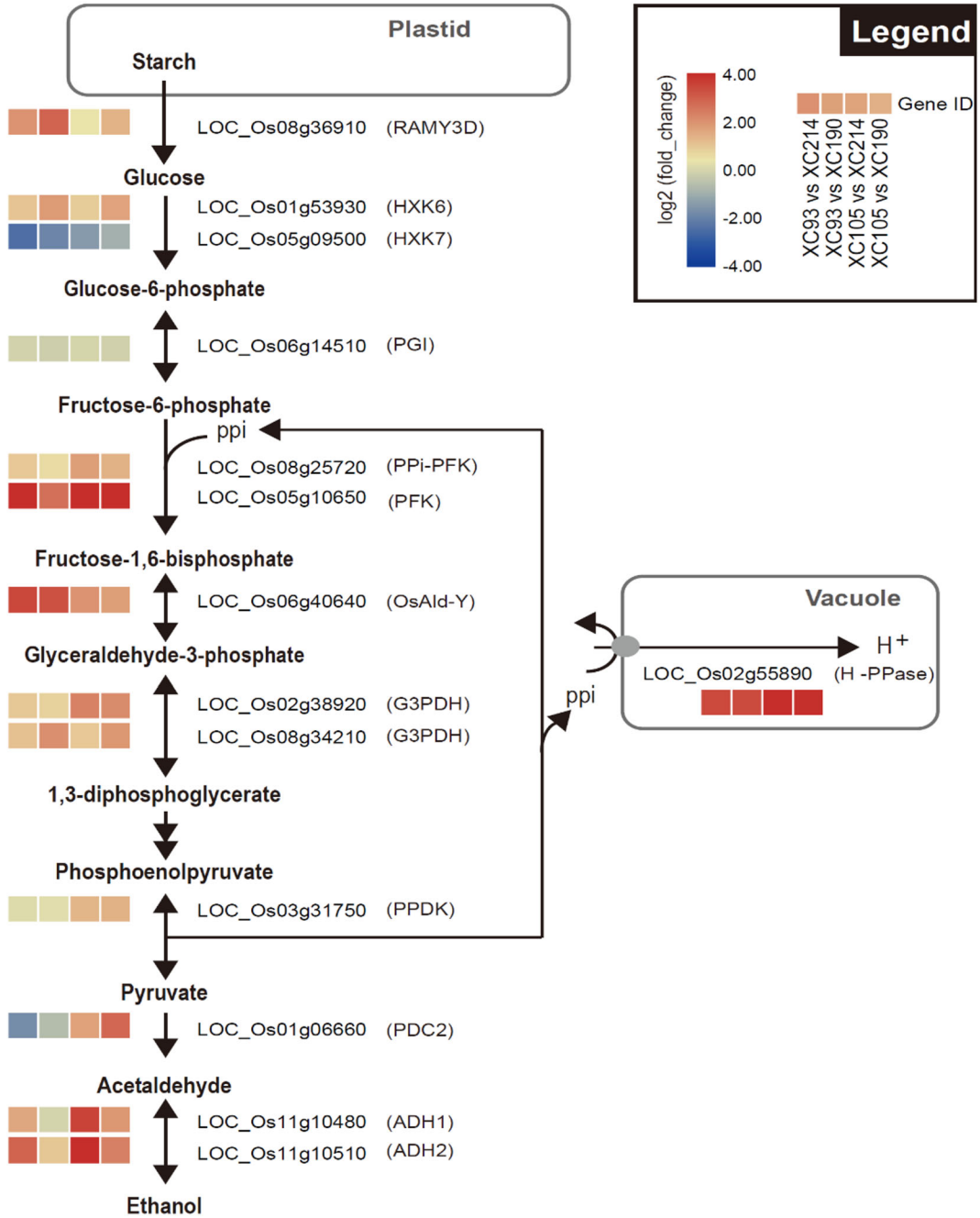


Fig. 5 Relative expression of genes involved in starch degradation, glycolysis, and ethanol fermentation between tolerant and sensitive genotypes. The pathway was modified from Hsu et al. (Hsu and Tung 2017)

dehydrogenase (ADH) genes, such as *LOC_Os11g10480* (*ADH1*) and *LOC_Os11g10510* (*ADH2*). Alcoholic fermentation was strongly activated during germination, which may help rice sustain energy under anoxia (Miro and Ismail 2013). The expression patterns of DEGs between tolerant and sensitive genotypes were confirmed in a previous single genome transcriptome study under submergence (Lasanthi-Kudahettige et al. 2007; Hsu and Tung 2015). It is suggested that the enhanced expression of these genes

enables tolerant varieties to maintain energy supply and support coleoptile growth.

Genes involved in cell elongation and ethylene and gibberellin signaling pathways

In addition to the identification of fundamental metabolism-related gene abundant in tolerant genotypes, DEGs that were involved in cell wall growth and loosening may explain the rapid growth of submerged coleoptiles of the tolerant genotypes. Here, we identified significant upregulation in several cell wall modification-related genes (Fig. S1, Table S5), including genes encoding the expansins (*LOC_Os01g60770*, *LOC_Os10g42610*) and the xyloglucan endotransglucosylase/hydrolase (*LOC_Os07g29750*, *LOC_Os11g33270*, *LOC_Os03g01800*, *LOC_Os06g48160*, *LOC_Os06g48180*). In addition, two ethylene transcription factor (ERF) genes (*LOC_Os09g11460*, *LOC_Os09g11480*) and some genes involved in gibberellin synthesis (*LOC_Os04g09900*, *LOC_Os06g37224*, *LOC_Os10g23160*) were significantly upregulated in tolerant types. These observations implied that genotypes with rapidly growing coleoptiles shared similar mechanisms related to ethylene or gibberellin signaling and cell wall modification which enable faster cell elongation to withstand and escape from submergence.

Then, we studied 26 genes involved in ethylene synthesis and 12 genes involved in gibberellin synthesis (Table S6). The results showed that 16 of the 26 genes involved in ethylene synthesis were upregulated. *LOC_Os08g30080* and *LOC_Os08g30100* were strongly induced in tolerant types (Fig. 6a), which encode ACC oxidase (ACO). Although four of the 12 genes involved in gibberellin synthesis were not expressed in both tolerance and sensitive types, the genes *LOC_Os02g36210*, *LOC_Os04g52230*, *LOC_Os06g37224*, and *LOC_Os10g23160* that respectively encode Ent-copalyl diphosphate synthase (CPS), ent-kaurene synthase (KS), ent-kaurene oxidase (KO), and ent-kaurenoic acid oxidase (KAO) were significantly upregulated in tolerant types (Fig. 6b).

Analysis of candidate genes and haplotype analysis of QTL genes in diverse germplasms

The loci identified by GWAS provided important clues for understanding the genotypic variation of rice submergence tolerance. We used LD decay values in rice to identify candidate genes responsible for each SCL, SSL, and STI locus. We extracted all genes within 100 kb of the most significant SNPs. To narrow down the candidate gene numbers, we considered data from RNA-seq analysis and the GO analysis (Yan et al. 2019); at the same time, we combined these results with known QTL regions.

Fifty candidate genes were found in 2160 DEGs (Fig. 7a). These candidate genes were located on *qAG1*-2, *qAG3*, *qAG5*, *qAG6*, *qAG-7-2*, *qAG11*, and *qAG12* (Table 2). Some of them were noteworthy. *LOC_Os01g59530* (*OsCML1*) and *LOC_Os12g41110* (*OsCML5*) were located in *qAG1-2* and *qAG12*, annotated as calmodulin. Ca^{2+} is a signal transducer during hypoxia and is also required for enzymes associated with glycolytic and ethanol fermentation in *Arabidopsis* and maize (Subbaiah et al. 1994; Sedbrook et al. 1996). In addition, four adjacent genes found on *qAG-11* (*LOC_Os11g47550*, *LOC_Os11g47570*, *LOC_Os11g47590*, and *LOC_Os11g47610*), all encoding glycosyl hydrolase, were significantly upregulated under submergence stress according to transcriptome data. Glycoside hydrolases catalyze the hydrolysis of glycosidic bonds in complex sugars such as cellulose and starch (Bourne and Henrissat 2001; Henrissat 1997). Compared with the sensitive type, the expressions of these genes were higher by hundreds-fold in tolerant ones. It was worth noting that the expression of these genes was also the same in the research of Lasanthi-Kudahettige and HSU (Table S7). Based on these results, we conducted further haplotype analysis of these four genes.

According to SNP data based on MSU7.0 annotation, we analyzed the haplotypes of these four genes. Totally, nine non-synonymous SNPs were detected at *LOC_Os11g47550*. Haplotype analysis revealed seven distinct haplotypes among these accessions, including two major haplotypes (Hap.1 and Hap.3). The result showed a large genetic difference between *indica* and *japonica* (Fig. 7b). Hap.1 was mostly present in *japonica*, while Hap.3 was mostly present in *indica*. Significant differences in SCL were detected between Hap.1 and Hap.3. Accessions with the Hap.1 genotype had longer SCL and SSL and higher STI than accessions having other haplotypes. Fifteen non-synonymous SNPs were detected at *LOC_Os11g47570*. Haplotype analysis revealed seven distinct haplotypes among these accessions, including four major haplotypes (Hap.1, Hap.2 and Hap.3, Hap.4). Hap.1 and Hap.2 were mostly

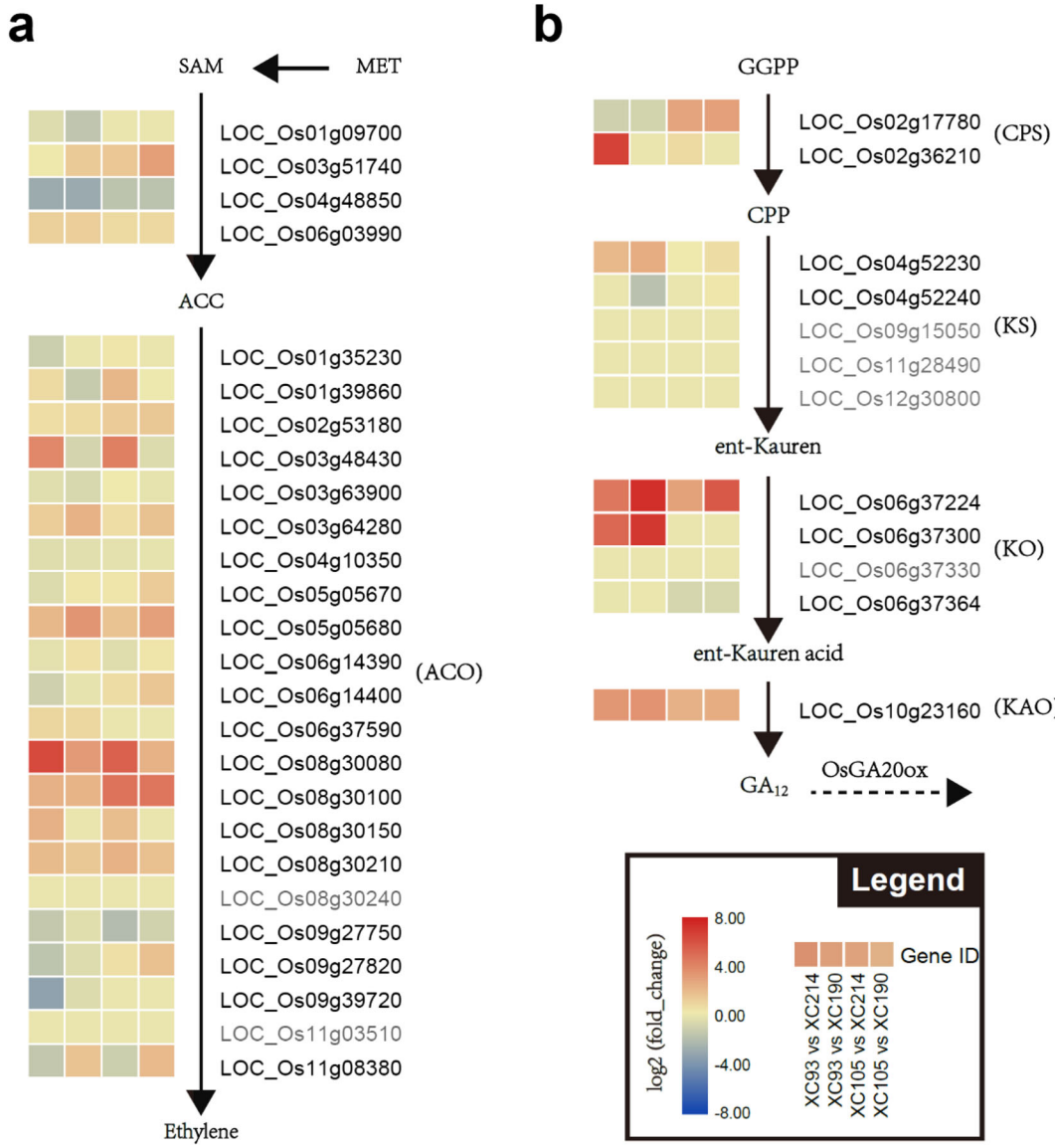
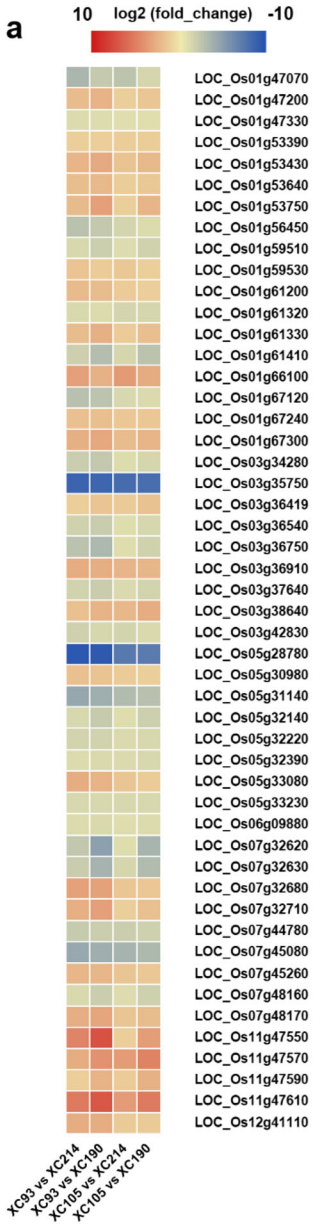


Fig. 6 Heatmap comparison showing the gene expression of the cultivars XC190, XC214, XC93, and XC105. **a** Genotypic expression of target genes involved in ethylene synthesis. **b** Genotypic expression of target genes involved in gibberellin synthesis. Genes in grey representation are not expressed in both tolerant and sensitive types. The pathway is simplified. ACO, ACC oxidase; CPS, Ent-copalyl diphosphate synthase; KS, Ent-kaurene synthase; KO, Ent-kaurene oxidase; KAO, Ent-kaurenoic acid oxidase

present in *japonica* rice, while Hap.3 and Hap.4 were mostly present in *indica* (Fig. 7c). Significant differences in SCL, SSL, and STI were detected between Hap.1, Hap.2 and Hap.3, Hap.4. Eight non-synonymous SNPs were detected at *LOC_Os11g47590*. Haplotype analysis revealed six distinct haplotypes among these accessions, including three major haplotypes (Hap.1, Hap.2, and Hap.3). Hap.1 was mostly present in *japonica*, while Hap.2 and Hap.3 were mostly present in *indica* (Fig. 7d). Significant differences in SCL, SSL, and STI were detected between Hap.1 and Hap.2, Hap.3. Eight non-synonymous SNPs were detected at *LOC_Os11g47610*. Haplotype analysis revealed four distinct haplotypes among these accessions, including two major haplotypes (Hap.1 and Hap.2) and a typically *ind-jap* differentiation was also observed among its haplotypes (Fig. 7e). Hap.1 was mostly present in *japonica*, while Hap.2 was mostly present in *indica*. Accessions with the Hap.1 genotype had longer SCL and SSL and higher STI.



b

Sub-population	Hap	Physical position (bp) 28734102	28734104	28736107	28736113	28736239	28736486	28736553	28736632	28736695	Num.	SCL	SSL	STI
Sub-J	Hap.1	C	T	G	T	T	C	G	C	G	42	3.37	6.18	3.27[a]
	Hap.2	T	T	G	T	T	C	G	C	G	5	3.40	6.22	3.26[a]
Sub-I	Hap.3	C	T	T	G	G	T	G	G	G	26	2.83	5.45	2.62[b]
	Hap.4	C	T	T	G	G	T	G	C	G	7	2.99	5.56	2.74[b]
	Hap.5	C	T	T	G	G	T	T	G	G	7	3.00	6.16	2.75[b]
	Hap.6	C	C	T	G	G	T	G	G	G	6	2.77	5.82	2.62[b]
	Hap.7	T	T	T	G	G	T	G	G	G	5	3.18	4.35	2.57[b]

c

Sub-population	Hap	Physical position (bp) 28748192	28748415	28748616	28748733	28748771	28748777	28748781	28748792	28748873	28748874	28748878	28748879	28750204	28750235	28750258	Num.	SCL	SSL	STI
Sub-J	Hap.1	G	T	G	C	C	A	A	G	C	T	C	A	T	T	G	42	3.32	5.92	3.19[a]
	Hap.2	T	T	G	C	C	A	A	G	C	T	C	A	T	T	G	13	3.43	5.34	3.24[a]
Sub-I	Hap.3	G	C	A	C	C	A	A	A	C	T	C	A	T	T	C	16	3.01	5.01	2.59[b]
	Hap.4	G	C	A	C	C	A	A	G	C	T	C	A	T	T	C	14	3.23	6.55	2.89[b]
	Hap.5	G	C	A	C	T	A	A	G	C	T	C	A	T	T	C	6	2.79	4.82	2.68[b]
	Hap.6	G	C	A	T	C	A	A	G	C	T	C	A	T	T	C	5	2.91	4.94	2.41[c]

d

Sub-population	Hap	Physical position (bp) 28754030	28754046	28754255	28754312	28754737	28756412	28756571	28756668	Num.	SCL	SSL	STI
Sub-J	Hap.1	C	G	A	A	A	G	A	G	37	3.38	5.15	3.12[a]
	Hap.2	C	A	A	A	A	G	A	G	7	3.32	5.72	3.26[a]
Sub-I	Hap.3	T	A	A	A	A	A	T	A	15	2.89	5.32	2.53[c]
	Hap.4	T	A	A	A	A	A	T	G	12	3.00	5.34	2.71[b]
	Hap.5	T	A	A	A	A	G	T	G	8	3.09	6.31	2.84[b]
	Hap.6	C	A	A	A	A	A	A	G	7	3.28	4.82	2.86[b]

e

Sub-population	Hap	Physical position (bp) 28759177	28759433	28759628	28759774	28759805	28759892	28759898	28759922	Num.	SCL	SSL	STI
Sub-J	Hap.1	T	C	C	C	G	T	G	C	52	3.36	5.59	3.17[a]
Sub-I	Hap.2	C	C	C	C	G	T	G	C	43	2.91	5.63	2.63[c]
	Hap.3	C	T	G	T	T	A	C	T	8	3.14	6.11	2.84[b]
	Hap.4	T	C	G	T	T	A	G	T	5	3.07	4.34	2.72[b]

Fig. 7 The relative expression of 50 candidate genes in four varieties and haplotype analysis of four QTL genes in diverse germplasms. **a** Heatmap comparison showing the relative expression of 50 candidate genes among four tolerant and sensitive varieties. **b** Haplotype analysis of *LOC_Os11g47550* and seedling submergence tolerance differences based on the haplotypes. **c** *LOC_Os11g47570*. **d** *LOC_Os11g47590*. **e** *LOC_Os11g47610*. Haplotype analysis shows only *indica* and *japonica* varieties (haplotypes with less than five accessions were omitted). The SNP data based on MSU7.0 annotation. The region containing coding region and 2-kb upstream of the gene was used to conduct haplotype analysis. $P < 0.05$ as significant difference

Discussion

Low seedling rate has always been the primary problem leading to low and unstable yield of direct seeding rice. The most sustainable method to maintain rice yield in the face of submergence disasters is cultivating submergence-tolerant cultivars (Angaji et al. 2010). The fast elongation of coleoptile could help improve the survival rate of rice seedlings in flooded soil (Yamauchi and Biswas 1997). Coleoptile elongation has been regarded as a major response to anaerobic stress (Magneschi et al. 2009; Kretzschmar et al. 2015;

Table 2 Determination of 50 candidate genes for submergence tolerance QTLs by integrated genomic and transcriptomic analysis. (Regulated (up/down): tolerant type vs sensitive type)

QTL	Gene ID	FPKM				Regulation (up/down)	Annotation
		XC105	XC93	XC214	XC190		
qAG1-2	*LOC_Os01g47070*	12.62	6.06	64.69	35.88	Down	Glycosyl hydrolase
	LOC_Os01g47200	5.26	4.07	1.02	1.88	Up	Retrotransposon protein putative unclassified
	LOC_Os01g47330	50.59	53.54	117.51	101.41	Down	Ribosomal protein L7/2FL12 C-terminal domain containing protein
	LOC_Os01g53390	21.35	22.60	10.46	9.78	Up	Glucosyltransferase
	LOC_Os01g53430	31.75	24.24	4.48	7.24	Up	Anthocyanidin 53-O-glucosyltransferase
	LOC_Os01g53640	19.56	17.93	4.60	7.66	Up	Retrotransposon protein putative unclassified
	LOC_Os01g53750	4.41	1.93	0.44	0.87	Up	Glucan endo-13-beta-glucosidase precursor
	LOC_Os01g56450	8.47	6.81	45.03	19.48	Down	DUF567 domain containing protein
	LOC_Os01g59510	12.79	21.13	52.49	43.79	Down	Chloroplast ribonuclease III domain protein
	LOC_Os01g59530	96.20	108.15	37.00	48.01	Up	OsCML1 - calmodulin-related calcium sensor protein
	LOC_Os01g61200	42.75	50.05	11.56	20.22	Up	GDSL-like lipase/2Facylhydrolase
	LOC_Os01g61320	5.99	6.08	14.41	17.26	Down	Thioredoxin
	LOC_Os01g61330	11.05	7.66	1.89	3.12	Up	Ankyrin homolog precursor
	LOC_Os01g61410	0.92	2.30	8.27	6.32	Down	NADH-ubiquinone oxidoreductase mitochondrial precursor
	LOC_Os01g66100	0.33	0.20	1.91	2.28	Down	Gibberellin 20 oxidase 2
	LOC_Os01g67120	27.66	27.26	187.73	67.31	Down	Rhodanese-like domain containing protein
	LOC_Os01g67240	10.92	11.60	3.16	4.06	Up	Formin-like protein 1 precursor
	LOC_Os01g67300	5.34	4.47	0.69	1.06	Up	Expressed protein
qAG3	*LOC_Os03g34280*	1.23	1.72	6.78	3.62	Down	Expressed protein
	LOC_Os03g35750	0.01	0.01	4.50	2.89	Down	Expressed protein
	LOC_Os03g36419	3.51	2.83	1.23	1.09	Up	Expressed protein
	LOC_Os03g36540	106.82	146.70	470.91	293.98	Down	Magnesium-chelatase subunit chlI chloroplast precursor
	LOC_Os03g36750	10.19	17.76	113.75	35.14	Down	cbbY
	LOC_Os03g36910	5.22	5.97	0.84	1.12	Up	SAM-dependent methyltransferase
	LOC_Os03g37640	32.88	48.01	148.37	106.17	Down	MATE efflux family protein
	LOC_Os03g38640	12.33	8.62	2.47	1.80	Up	Expressed protein
	LOC_Os03g42830	0.31	0.26	0.86	0.77	Down	MATE efflux family protein
qAG5	*LOC_Os05g28780*	0.01	0.01	7.71	1.63	Down	GCRP10 - Glycine and cysteine-rich family protein precursor
	LOC_Os05g30980	30.83	37.31	10.25	14.97	Up	RNA recognition motif containing protein
	LOC_Os05g31140	4.65	3.86	82.59	34.34	Down	Glycosyl hydrolases family 17
	LOC_Os05g32140	38.22	74.06	182.28	144.05	Down	NAD dependent epimerase/2Fdehydratase family protein
	LOC_Os05g32220	81.27	95.51	282.59	216.61	Down	L1P family of ribosomal proteins domain containing protein
	LOC_Os05g32390	8.30	9.93	21.23	22.27	Down	FZL
	LOC_Os05g33080	12.02	16.63	2.29	5.40	Up	Serine/2Fthreonine-protein kinase
	LOC_Os05g33230	3.51	3.88	9.47	9.43	Down	Cobalt ion transporter

Table 2 (continued)

QTL	Gene ID	FPKM				Regulation (up/down)	Annotation
		XC105	XC93	XC214	XC190		
qAG6	*LOC_Os06g09880*	1.31	1.59	3.41	3.11	Down	EMB1270
qAG-7-2	*LOC_Os07g32620*	0.09	0.61	3.07	1.21	Down	Anthocyanidin 53-O-glucosyltransferase
	LOC_Os07g32630	0.29	0.99	3.97	2.62	Down	UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein
	LOC_Os07g32680	67.58	75.77	7.15	25.13	Up	Retrotransposon protein putative unclassified
	LOC_Os07g32710	114.68	75.70	11.58	34.90	Up	Retrotransposon protein putative unclassified
	LOC_Os07g44780	4.43	4.28	18.98	16.16	Down	GDSL-like lipase/2Facylhydrolase
	LOC_Os07g45080	37.14	31.13	644.42	390.85	Down	Expressed protein
	LOC_Os07g45260	4.25	4.89	0.94	1.57	Up	Glycosyl transferase 8 domain containing protein
	LOC_Os07g48160	5.96	10.76	25.35	21.94	Down	Alpha-galactosidase precursor
	LOC_Os07g48170	0.78	0.65	0.10	0.21	Up	Nucleotidyltransferase
qAG11	*LOC_Os11g47550*	240.38	47.11	1.63	20.79	Up	Glycosyl hydrolase
	LOC_Os11g47570	450.83	205.24	28.33	16.31	Up	Glycosyl hydrolase
	LOC_Os11g47590	155.49	66.69	29.72	27.06	Up	Glycosyl hydrolase
	LOC_Os11g47610	107.08	35.37	0.88	2.78	Up	Glycosyl hydrolase
qAG12	*LOC_Os12g41110*	30.96	33.33	4.63	12.88	Up	OsCML5 - Calmodulin-related calcium sensor protein

Setter and Ella 1994; Gibbs et al. 2000; Nagai et al. 2010; Lasanthi-Kudahettige et al. 2007). The rapid elongation of coleoptile could help plants break through the water surface to obtain oxygen, which is beneficial to the formation of rice plant morphology under anoxic conditions. In this study, the SCL, SSL, and STI were determined in the submergence environment. It was found that there was a significantly positive correlation between coleoptile elongation and rice submergence tolerance, which was the same as previous studies (Zhang et al. 2017; Hsu and Tung 2015). Therefore, fast developed coleoptile is an important characteristic of rice suitable for direct seeding technology. It is of great importance to mine genetic loci controlling this trait.

Based on the GWAS results, several anaerobic germination-associated loci were identified. Some were novel discoveries and some were co-localized with genomic intervals reported in previous biparental QTL studies. Previous studies have shown that multiple intervals at chromosomes 3, 7, and 11 are associated with seedling survival or shoot elongation (Fukao and Bailey-Serres 2008; Kang et al. 2010; Septiningsih et al. 2013; Hsu and Tung 2015; Lv et al. 2017). In this study, we detected QTLs on chromosomes 3, 7, and 11 in three groups of phenotypes (SCL, SSL, and STI) by GWAS, which indicated that there were multiple allele variations in the reported QTLs. In addition, we have detected some SNPs close to known genes. *RAMY3D* and *OsTPP7* were detected significantly close to SNPs in different GWAS groups. Because many SNPs around this gene were still not detected, we inferred that local LD might cause a misleading association and lead to a large distance. Then, we compared the FPKM values of these genes among different varieties. The expressions of *RAMY3D* in tolerant types were higher than those in sensitive ones. *OsTPP7*'s expression was not detected in XC214, and the expression level in XC105 was higher than that in XC190.

The elongation of submerged coleoptile depends mainly on the elongation of cells. Several genes encoding cell wall loosening proteins, such as expansins, have been shown to be uniquely expressed under anoxia (Lasanthi-Kudahettige et al. 2007). Based on the previous study (Cosgrove 2005; Hsu and Tung

2017), we analyzed the expression of 20 genes involved in cell wall growth and loosening. The genes (*LOC_Os02g18650*, *LOC_Os04g51340*, *LOC_Os04g46630*, *LOC_Os06g48160*, and *LOC_Os06g48180*) were significantly upregulated in tolerance type. Although most of them were upregulated, we still found that the expressions of several genes were different from those of previous studies. In our study, *LOC_Os01g20980* and *LOC_Os10g39640* were downregulated in the tolerance type but upregulated in the previous study. Due to the complexity of transcriptomic fine-tuning in response to submergence in rice seedlings from diverse genotypes (Hsu and Tung 2017), the genes were differentially expressed among diverse genotypes.

Ethylene and gibberellin play an important role in submergence-tolerant rice (Watanabe et al. 2007). Ethylene is an important signaling phytohormone that quickly accumulates in submerged plants. Additionally, it stimulates multiple downstream mechanisms (Fukao and Bailey-Serres 2008). Previous studies have demonstrated increased ethylene accumulation in young seedlings of the submergence-tolerant rice variety "Khao Hlan On" in comparison with the sensitive IR42, which suggests that ethylene biosynthesis genes are involved in submergence tolerance during germination (Ismail et al. 2009). Gibberellin helps cells elongate under water (Bailey-Serres and Voesenek 2010). Hence, we studied 26 genes involved in ethylene synthesis and 12 genes involved in gibberellin synthesis. The results showed that most of them were upregulated in tolerant types. In addition, two ethylene-responsive factor (ERF) genes, *LOC_Os09g11460* (*Sub1B*) and *LOC_Os09g11480* (*Sub1C*), were induced in all tolerant genotypes. *LOC_Os01g66100* (*sd1*) is a candidate gene we detected, and it was also upregulated in tolerant types. *sd1* encodes a key enzyme in gibberellin synthesis, GA20ox. The *sd1* protein directs increased synthesis of gibberellins, largely GA_4, which promotes internode elongation. The expression of *sd1* in deep-water rice could make rice internodes extend rapidly after 10 leaf stage, to help the leaves of rice come out water quickly and get oxygen (Kuroha et al. 2018).

Whether the varieties with longer coleoptile or higher emergence rate were artificially selected is another point worth exploring. A previous study identified two major *indica* subpopulations based on the low-coverage sequencing data of 1479 rice accessions, *ind I* and *ind II*, and found 200 regions that were differentially selected between *ind I* and *ind II* (Xie et al. 2015). Here, 10 candidate genes (*LOC_Os01g53390*, *LOC_Os01g53640*, *LOC_Os01g53750*, *LOC_Os01g56450*, *LOC_Os01g61200*, *LOC_Os01g61320*, *LOC_Os01g61330*, *LOC_Os01g66100*, *LOC_Os07g44780*, and *LOC_Os07g45080*) were located in these selected regions. Some of these genes have been reported to be involved in other abiotic stresses in rice. It is suggested that these genes may inadvertently become breeding targets in the process of artificial breeding. These results provide new ideas for future research.

Clearly, several metabolic processes are involved in rice submergence tolerance, which are coordinated in a manner that facilitates germination and fast growth of the embryo to emerge from flooded soils (Miro and Ismail 2013). Rice coleoptile elongation is the result of multiple gene interactions. Under submergence or anoxic conditions, most of the genes cannot be expressed normally, which makes rice express some genes related to stress. These genes participate in the coleoptile elongation of rice at seedling stage to help rice emerge from water as soon as possible and obtain oxygen. In this study, we found some candidate genes by GWAS and verified by transcriptomic analysis. Based on GWAS localization results, transcriptomic analysis, reported expression profile data, and the information of genes function annotation, we selected *LOC_Os11g47550*, *LOC_Os11g47570*, *LOC_Os11g47590*, and *LOC_Os11g47610* for haplotype analysis. Further analysis suggested these genes showed a large genetic difference in diverse germplasms. Based on the diverse germplasms, we determined the natural haplotype of these genes. These results help us to better understand the molecular mechanism of the difference in tolerance of diverse germplasms to submergence; meanwhile, it provided materials and more choices for further selection of new submergence tolerant varieties.

Conclusions

In this study, we used GWAS to identify the candidate genes controlling rice coleoptile elongation under submergence conditions and verified them by transcriptomic analysis. The significant genomic regions including *LOC_Os11g47550*, *LOC_Os11g47570*, *LOC_Os11g47590*, and *LOC_Os11g47610* are potential candidates that should

be studied further and incorporated into elite rice cultivars to improve seedling survival during anaerobic germination.

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Data availability All data generated or analyzed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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