

Article

Stage- and Temperature-Dependent Coleoptile Elongation Under Submergence in Thai *indica* Rice (*Oryza sativa* L.)

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Abstract

Direct-seeded rice offers agronomic and economic advantages, but early-season flooding creates hypoxic conditions around germinating seeds and young seedlings, limiting crop establishment. Understanding genotypic variation in early submergence responses is therefore important for improving tolerance during anaerobic germination (AG) and anaerobic seedling development (ASD). This study aimed to characterize submerged coleoptile elongation in Thai rice germplasm and identify genetic factors associated with this response. We screened 192 Thai *indica* rice accessions with submergence imposed before or after germination to evaluate AG and ASD responses under naturally varying ambient temperatures. Underwater coleoptile length and elongation index showed substantial genotypic variation, while responses under AG and ASD were only weakly correlated ($r = 0.29$), indicating that tolerance differs between developmental stages. Across trials, coleoptile elongation under submergence was strongly influenced by the environment, with higher ambient temperature associated with reduced elongation and genotype-dependent sensitivity to the ambient temperature–dissolved oxygen (DO) gradient. Controlled-temperature experiments further showed that elevated temperature was associated with lower DO concentrations and reduced underwater coleoptile elongation. Genome-wide association analysis using mixed-model-adjusted phenotypes identified four quantitative trait loci (QTLs) for coleoptile length and three for elongation index under AG, along with one QTL for coleoptile length under ASD. Integrative analysis of a chromosome 12 QTL combining local linkage disequilibrium, public RNA-seq data, and haplotype variation prioritized *OsSWEET13*, *OsNAM*, and *OsARF24* as candidate genes for coleoptile elongation under submergence. Together, these findings show that developmental stage, temperature, and genetic background shape submerged coleoptile elongation in Thai *indica* rice.

Keywords: anaerobic germination; climate change; direct-seeded rice; genome-wide association study (GWAS); rice



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1. Introduction

Direct-seeded rice (DSR) is a crop establishment system in which seeds are sown directly into the field, eliminating nursery raising and transplanting. DSR is commonly categorized into dry-DSR (dry seed sown into dry soil), wet-DSR (pre-germinated seed sown into puddled soil), and water-DSR (dry or pre-germinated seed broadcast into standing water) [1]. Compared with transplanting, DSR reduces water use, labor and production costs, and crop duration while improving resource-use efficiency, and water-DSR can additionally suppress weeds, lowering herbicide inputs [2]. Despite these advantages, stand establishment remains a major constraint, particularly under erratic rainfall and inadequate field drainage that expose seeds and young seedlings to transient or prolonged flooding.

Flooding at sowing imposes low-oxygen (O_2) stress that restricts seedling emergence and establishment. Although rice can germinate under hypoxic conditions, most cultivated and modern varieties are sensitive to early-stage submergence [3], a risk expected to increase as climate change intensifies rainfall variability and flooding events. Anaerobic germination (AG) and anaerobic seedling development (ASD), defined as the ability of seeds to germinate and seedlings to establish under O_2 -limited conditions, are therefore key traits for stabilizing DSR productivity. Multiple traits have been associated with AG/ASD tolerance, including germinability, coleoptile morphology (length, diameter, surface area), seedling survival rate, and anaerobic response/tolerance indices [4–6]. Among these, coleoptile elongation is particularly informative because it enables seedlings to approach the water surface, enhancing internal aeration and supporting efficient carbohydrate mobilization for early growth [7]. Accordingly, greater coleoptile elongation is generally advantageous for seedling survival and establishment under AG/ASD. Tolerant genotypes often couple rapid coleoptile extension with delayed radicle development, prioritizing a shoot escape strategy under submergence, whereas sensitive genotypes with poor elongation frequently fail to establish seedlings [8,9].

Rapid coleoptile extension is driven primarily by cell elongation, supported by auxin- and gibberellin (GA)-mediated signaling and increased cell-wall extensibility [10]. Auxin biosynthesis and signaling are upregulated during AG. Genetic or chemical perturbation of auxin biosynthesis, transport, or signaling substantially reduces coleoptile growth, while enhanced auxin activity promotes submerged coleoptile elongation [11–14]. Suppression of the microRNA miR393 under submergence stabilizes the auxin receptors TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/AUXIN SIGNALING F-BOX 2 (AFB2), activating the auxin signaling pathway and AUXIN RESPONSE FACTOR (ARF) expression and inducing submerged coleoptile elongation [15]. Continued growth also requires efficient mobilization of endosperm carbohydrate reserves by starch-hydrolyzing enzymes and their delivery to growing coleoptile by sugar transporters, together with the maintenance of energy production under O_2 limitation [14,16]. GA promotes starch breakdown via the R2R3-MYB transcription factor GAMYB, which activates α -amylase expression [17], while O_2 - and sugar-starvation signaling activates additional α -amylases through the transcription factor MYBS1, regulated by the CIPK15–SnRK1A cascade [18]. Starch degradation, glycolysis, and alcoholic fermentation collectively provide soluble sugars and ATP needed to sustain elongation, while aldehyde detoxification and redox regulation help maintain cellular function [17–20]. These coordinated hormonal and metabolic responses enable tolerant seedlings to extend their coleoptiles toward the water surface, where improved internal aeration supports subsequent growth.

Genetic studies have identified several key loci that connect variation in AG and submerged seedling development with the underlying tolerance mechanisms [21]. The major quantitative trait locus (QTL) *qAG-9-2/AG1* contains *TREHALOSE-6-PHOSPHATE PHOSPHATASE 7* (*OsTPP7*), which promotes carbon allocation and energy availability

under O₂ limitation, whereas a chromosome 1 QTL implicating *HEXOKINASE 6* (*OsHKK6*) further highlights the importance of sugar sensing and metabolism [6,22]. Genome-wide association studies (GWASs) have extended these findings by identifying loci involved in glycolysis and carbohydrate metabolism, including *EARLY EMBRYOGENESIS-SPECIFIC ENOLASE 1* (*OsEE1*), as well as transcriptional regulators such as *OsDREB6* that coordinate sucrose- and starch-related responses [5,23]. Additional loci involved in abscisic acid, jasmonic acid, and ethylene signaling indicate that complex hormonal regulation contributes to variation in germination and coleoptile elongation under submergence. Moreover, the identification of *CLASSY 1* (*OsCLSY1*), a component of RNA-directed DNA methylation, suggests that epigenetic regulation may influence submerged seedling development in a genetic background-dependent manner [24–30]. These studies show that AG and ASD tolerance is polygenic and depends on the coordinated regulation of carbohydrate mobilization, energy production, cell elongation, hormonal signaling, and gene expression under O₂-limited conditions. These QTLs and genes, together with improved understanding of the AG and ASD mechanisms, provide valuable resources for marker-assisted introgression and pyramiding of favorable loci into elite rice varieties to improve establishment under submergence [14,21].

DSR is widely practiced in Thailand and is expected to expand as increasing mechanization addresses labor shortages associated with population aging [31]. Although AG tolerance has been evaluated in a limited number of Thai rice accessions [32], the diversity of early submergence responses and their genetic basis in Thai germplasm remain largely uncharacterized. Moreover, most previous studies have evaluated tolerance using either dry seeds or pre-germinated seeds, leaving the consistency of genotypic responses across developmental stages unresolved. Because pre-germinated seeds are commonly used in wet- and water-DSR, comparing submergence imposed before germination with that imposed after radicle emergence provides a practical framework for distinguishing tolerance during AG and ASD. Such a comparison may reveal whether genotypes that perform well during germination also maintain vigorous coleoptile elongation when submergence occurs after germination.

In this study, we screened underwater coleoptile elongation in 192 Thai *indica* rice accessions by imposing submergence either before germination or after radicle emergence to distinguish AG and ASD responses. Screening under natural ambient environmental conditions allowed us to assess phenotypic diversity, the consistency of genotypic performance across developmental stages, and environmental variation in coleoptile elongation. We subsequently conducted controlled-temperature experiments to examine the relationships among temperature, dissolved O₂ (DO) dynamics, and underwater elongation. As a complementary genetic analysis, we performed GWAS using mixed-model-adjusted phenotypes to account for experimental design and environmental heterogeneity and identify genomic regions associated with coleoptile elongation under AG and ASD. This study provides a broad characterization of early submergence responses in Thai *indica* rice, clarifies the developmental and environmental sensitivity of underwater coleoptile elongation, and identifies germplasm and genomic regions useful for mechanistic studies and the improvement of rice establishment under flood-prone DSR conditions.

2. Results

2.1. Variation in Coleoptile Elongation During AG Among Thai Indica Rice Accessions

To characterize variation in coleoptile elongation during AG, we evaluated coleoptile growth under aerobic and submerged conditions in a panel of 192 Thai *indica* rice accessions (Figure 1A; Table S1). The panel included the well-known aromatic cultivar ‘Khao Dawk Mali 105’ (KDML105) and its improved derivative ‘Pathumthani 1’ (PTT1). The *japonica*

variety Nipponbare and the *indica* variety IR64 were included as AG-tolerant and AG-sensitive reference varieties, respectively [6]. Imbibed seeds were germinated in darkness for 7 days under either aerobic or submerged conditions across four independent trials conducted under naturally varying ambient temperatures. Germination and coleoptile length were evaluated at the end of each trial. Because temperature was not experimentally controlled, it varied both within and among trials.

Across the panel, mean germination (averaged across four trials) ranged from 80.01 to 100% under aerobic conditions (panel mean = 94.57%) and from 68.32 to 100% under submergence (panel mean = 92.15%), with no significant difference between conditions for any genotype (Figure S1A; Table 1). Under aerobic conditions, seedlings underwent normal leaf emergence and produced short coleoptiles ranging from 8.87 to 15.57 mm (Figure 1B; Table 1). In contrast, submergence promoted coleoptile elongation to varying degrees across genotypes, with coleoptile lengths ranging from 26.50 to 57.75 mm and a panel mean of 40.05 mm (Figure 1B; Table 1). Several Thai accessions produced coleoptiles comparable in length to or longer than those of Nipponbare (Figure 1B). Nevertheless, more than 76% of the panel (146 accessions), including KDML105 and PTT1, produced coleoptiles comparable in length to or shorter than those of IR64 (mean coleoptile length 43.71 mm), indicating that strong elongation responses were uncommon within the panel.

Table 1. Estimates of variance components, genetic variability, heritability, and genetic advance for germination, coleoptile length, and elongation index in 192 Thai rice accessions.

Condition	Aerobic Control		AG			ASD	
Trait	Germination (%)	Coleoptile Length (mm)	Germination (%)	Coleoptile Length (mm)	Elongation Index	Germination (%)	Coleoptile Length (mm)
Range	80.01–100	8.87–15.57	68.32–100	26.50–57.75	1.18–4.14	61.88–100	27.06–51.51
Mean $\pm$ SD	94.57 $\pm$ 3.22	11.67 $\pm$ 1.25	92.15 $\pm$ 4.16	40.05 $\pm$ 5.86	2.48 $\pm$ 0.52	92.48 $\pm$ 4.36	39.14 $\pm$ 4.70
σ_g^2	5.16	1.52	10.95	21.24	0.15	14.03	17.08
σ_p^2	10.35	1.84	17.27	34.03	0.27	19.01	21.33
σ_e^2	20.73	4.11	25.29	77.27	0.48	19.93	79.67
GCV (%)	2.40	10.56	3.59	11.51	15.81	4.05	10.36
PCV (%)	3.40	11.62	4.51	14.57	21.11	4.71	11.58
PCV-GCV	1.00	1.06	0.92	3.06	5.30	0.66	1.22
H^2 (%)	49.91	82.57	63.41	62.43	56.10	73.80	80.05
GAM	3.50	19.77	5.89	18.73	24.40	7.17	19.10

Notes: These parameters were estimated across 192 Thai rice cultivars based on four independent trials. SD, standard deviation; σ_g^2 , genotypic variance; σ_p^2 , phenotypic variance; σ_e^2 , the residual variance; GCV, genotypic coefficient of variation; PCV, phenotypic coefficient of variation; H^2 , broad-sense heritability; GAM, genetic advance as percentage of the mean. σ_e^2 is the residual variance from the mixed model prior to averaging over replicates.

We next quantified the coleoptile elongation response of each genotype using an elongation index, defined as the proportional increase in coleoptile length under submergence relative to aerobic conditions. The AG-tolerant reference Nipponbare exhibited the highest elongation response, with a mean index of 4.51. Among the 192 Thai accessions, ‘Leuang Awn Bow’ displayed the highest index (4.14), whereas PTT1 had the lowest (1.18) (Figure 1C; Table 1). Based on coleoptile length and elongation index, we identified Thai accessions with contrasting elongation responses during AG (Figure 1D), providing genetic resources for comparative physiological studies, genetic analyses, and the improvement of early rice establishment under submergence.

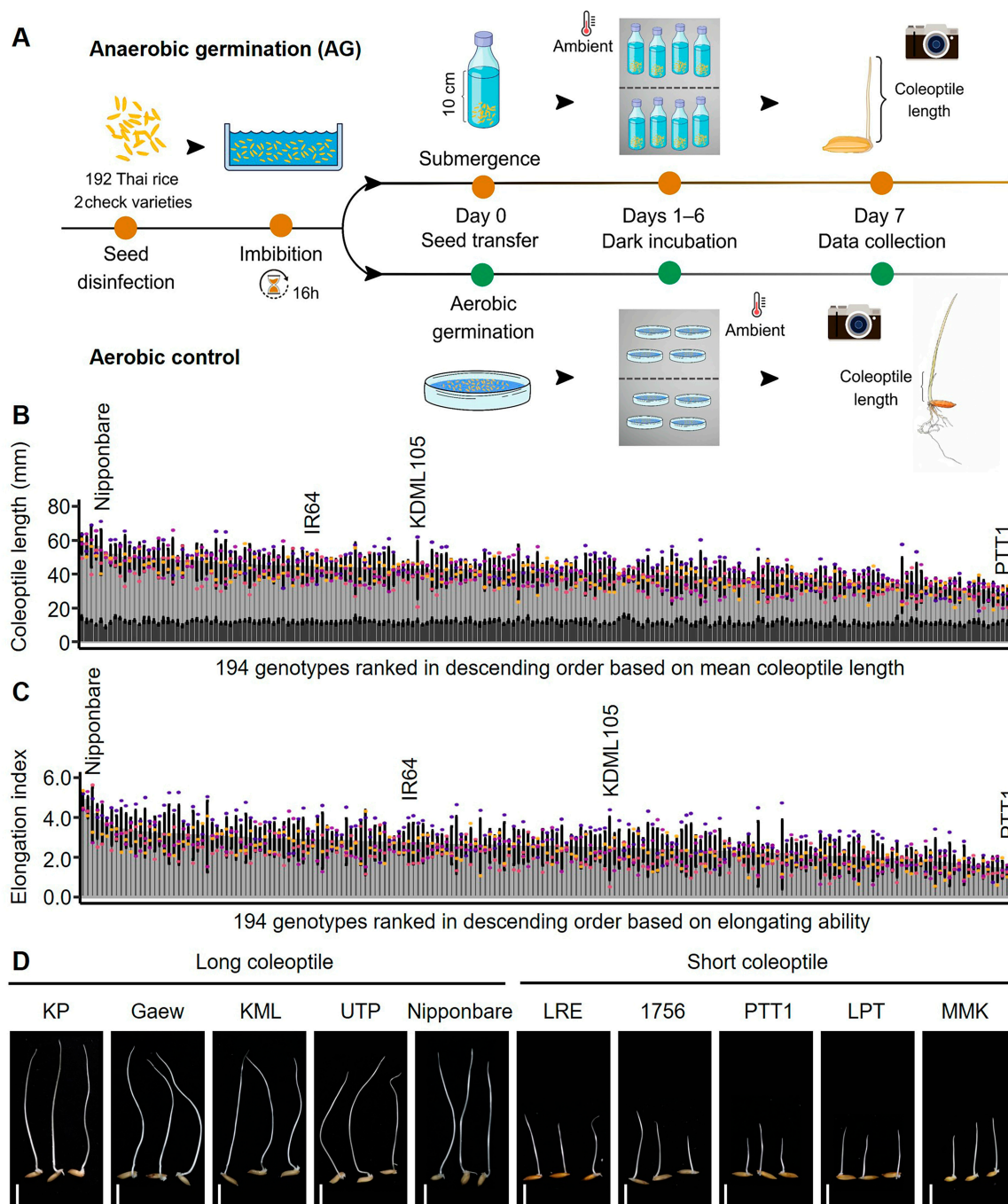


Figure 1. Genotypic diversity in coleoptile elongation responses under aerobic and AG conditions. (A) Schematic of the experimental setup used to evaluate coleoptile elongation under AG and aerobic control conditions. (B) Bar plots showing variation in coleoptile length under aerobic control (dark gray) and AG conditions (light gray) across 194 rice genotypes, comprising 192 Thai *indica* accessions and the reference varieties Nipponbare and IR64. The aromatic cultivars KDML105 and PTT1 and the reference varieties Nipponbare and IR64 are highlighted. (C) Coleoptile elongation index of the genotypes shown in (B), calculated from coleoptile lengths under AG relative to aerobic conditions. (D) Representative images of rice accessions with contrasting coleoptile elongation responses during AG, based on coleoptile length and elongation index. Four Thai accessions with strong elongation responses, ‘Khao Prae’ (KP), Gaew, ‘Khao Maled Lek’ (KML), and ‘U-tapao’ (UTP), are shown alongside Nipponbare. Scale bars = 10 mm. Data in (B,C) are presented as mean $\pm$ SD from four independent trials ($n = 4$). Accessions are sorted in descending order according to the mean value of the trait shown in each panel; dots represent individual trial means, with different colors representing the four independent trials.

2.2. Coleoptile Elongation Responses Differ Between AG and ASD

Because pre-germinated seeds are commonly used in wet- and water-DSR, we examined whether underwater coleoptile elongation differed when submergence was imposed after germination. Imbibed seeds were first incubated under aerobic conditions for 30 h to allow radicle emergence and were then submerged for 5 days at naturally varying ambient temperatures (Figure 2A). This treatment was used to evaluate ASD responses, in contrast to AG, in which submergence was imposed before germination.

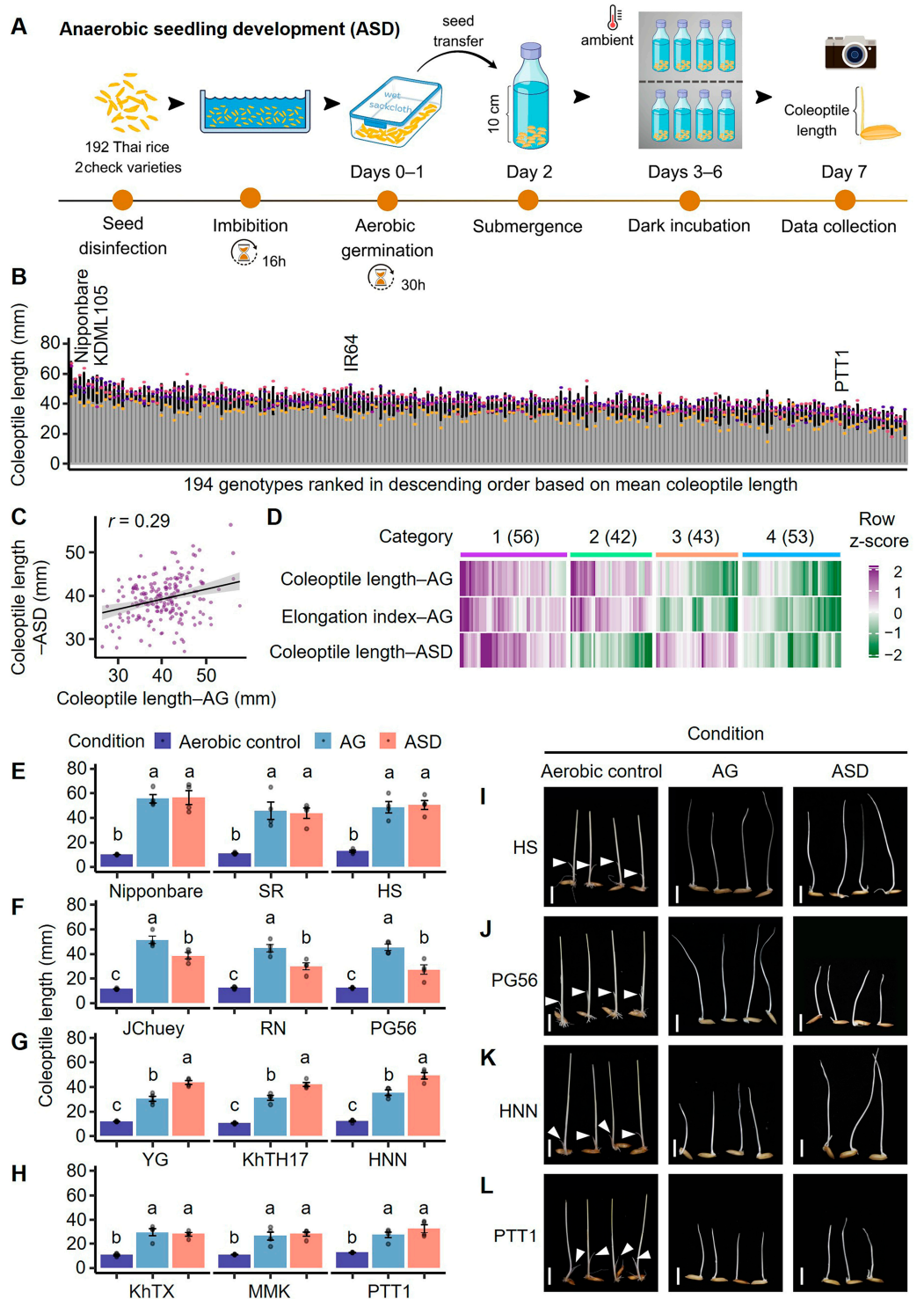


Figure 2. Developmental stage-dependent variation in coleoptile elongation among rice genotypes. (A) Schematic of the experimental setup used to evaluate coleoptile elongation during ASD.

(B) Variation in coleoptile length under ASD among 194 rice genotypes, comprising 192 Thai *indica* accessions and the reference varieties Nipponbare and IR64. Nipponbare, IR64, KDML105, and PTT1 are highlighted. Data are presented as mean $\pm$ SD from four independent trials ($n = 4$), with genotypes sorted in descending order according to mean coleoptile length under ASD; dots represent individual trial means, with different colors representing the four independent trials. (C) Relationship between coleoptile length during AG and ASD across genotypes. The value of r denotes Pearson's correlation coefficient. (D) Heatmap showing partitioning around medoids (PAM) clustering of genotypes based on coleoptile length and elongation index during AG and coleoptile length during ASD. Genotypes were separated into four response groups: relatively strong elongation during both AG and ASD (1), primarily during AG (2), primarily during ASD (3), and relatively weak elongation during both treatments (4). Numbers in parentheses indicate the number of genotypes in each group. (E–L) Quantification and representative images of coleoptile elongation under aerobic control, AG, and ASD conditions for selected genotypes from each response group: (E,I) strong elongation during both AG and ASD (Nipponbare, SR, and HS); (F,J) stronger elongation during AG than ASD (JChuey, RN, and PG56); (G,K) stronger elongation during ASD than AG (YG, KhTH17, and HNN); and (H,L) weak elongation during both AG and ASD (KhTX, MMK, and PTT1). Data in (E–H) are presented as mean $\pm$ SD from four independent trials ($n = 4$); dots represent individual trial means. Within each genotype, different letters indicate significant differences among conditions according to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT; $p < 0.05$). Scale bars in (I–L) = 10 mm. Arrowheads indicate coleoptiles under aerobic control conditions.

Across the 194 rice accessions, germination percentages following the ASD treatment were comparable to those observed under aerobic and AG conditions (Figure S1B–E; Table 1). Although radicles had emerged before submergence, subsequent underwater growth was characterized by limited radicle extension and pronounced coleoptile elongation across genotypes. Nipponbare produced the longest coleoptile under ASD (56.36 mm), whereas coleoptile lengths among the Thai accessions ranged from 27.06 mm in 'Pin Gaew 56' (PG56) to 51.51 mm in 'Maled Lek Bow' (Figure 2B; Table 1).

Coleoptile length under ASD was only weakly correlated with that under AG across the panel ($r = 0.29$, $p < 0.01$; Figure 2C), indicating limited consistency in genotypic elongation responses between the two developmental stages. Clustering based on coleoptile length and elongation index during AG, together with coleoptile length during ASD, separated the genotypes into four response groups (Figure 2D). Of these, 56 genotypes showed relatively strong elongation under both AG and ASD, whereas 42 showed stronger elongation primarily during AG, 43 primarily during ASD, and 53 showed relatively weak elongation under both conditions. Representative genotypes included Nipponbare, 'Sawng Ruang' (SR), and 'Hawm Surin' (HS), which showed strong elongation under both treatments (Figure 2E,I); 'Jek Chuey' (JChuey), 'Rawd Ni' (RN), and PG56, which showed stronger responses during AG than ASD (Figure 2F,J); 'Yai Graeng' (YG), 'Khao Tah Haeng 17' (KhTH17), and 'Hawm Nahng Nuan' (HNN), which showed stronger responses during ASD than AG (Figure 2G,K); and 'Khao Tah Ex' (KhTX), 'Med Ma Kheua' (MMK), and PTT1, which showed weak elongation under both treatments (Figure 2H,L). These results demonstrate that underwater coleoptile elongation is developmentally dependent and that responses measured during AG do not necessarily predict those observed during ASD.

2.3. Genetic and Environmental Contributions to Variation in Coleoptile Elongation

Genetic parameters for germination and coleoptile elongation under aerobic, AG, and ASD conditions are summarized in Table 1. Under aerobic conditions, germination percentage and coleoptile length showed limited phenotypic dispersion and relatively low genotypic variances ($\sigma_g^2 = 5.16$ and 1.52, respectively). Germination percentage exhibited moderate heritability ($H^2 = 49.91\%$) but low genetic advance as a percentage of the mean (GAM = 3.50%). In contrast, coleoptile length showed high heritability ($H^2 = 82.57\%$) and a

small difference between the phenotypic and genotypic coefficients of variation (PCV–GCV = 1.06 percentage points), indicating that most of the observed variation under aerobic conditions was attributable to genotypic differences.

Under AG, coleoptile length showed greater variability ($\sigma_g^2 = 21.24$; $\sigma_p^2 = 34.03$), moderate genotypic variation (GCV = 11.51%), and moderate heritability ($H^2 = 62.43\%$). Its GAM was 18.73%, while the wider PCV–GCV difference of 3.06 percentage points indicated a greater environmental contribution than under aerobic conditions. The elongation index showed the highest GCV (15.81%) and GAM (24.40%) among the evaluated traits, but also the widest PCV–GCV difference (5.30 percentage points) and lower heritability ($H^2 = 56.10\%$), indicating comparatively greater sensitivity to non-genetic variation.

Under ASD, coleoptile length showed high heritability ($H^2 = 80.05\%$), a small PCV–GCV difference of 1.22 percentage points, and moderate GAM (19.10%). By comparison, germination percentage under AG and ASD showed moderate-to-high heritability ($H^2 = 63.41\%$ and 73.80% , respectively) but low GCV (3.59% and 4.05%) and GAM (5.89% and 7.17%), indicating the limited variation in germination across the panel. Overall, coleoptile length under submergence contained substantial genotypic variation under both AG and ASD, although the relative contribution of environmental variation was greater during AG.

2.4. Coleoptile Elongation Varies with Ambient Temperature and Dissolved O₂ Conditions

Pronounced within-genotype variation in coleoptile elongation was observed among independent trials of both the AG and ASD assays. During AG, one trial produced consistently longer coleoptiles across much of the panel (Figure 1B,C), whereas during ASD, another trial produced generally shorter coleoptiles (Figure 2B). Given that phenotyping was conducted under natural environmental conditions, air and water temperatures were not experimentally controlled or maintained at constant levels and varied both within and among trials. Ambient temperatures were recorded throughout the experiments, allowing us to examine whether the observed trial-to-trial variation in coleoptile responses was associated with differences in temperature. Trials were therefore ordered according to their mean ambient temperature.

During AG, ambient temperature differed among trials, with daily ranges increasing from 29.65–31.98 °C in trial 1 to 31.06–33.64 °C in trial 4, and mean temperatures increasing from 30.56 °C to 32.22 °C (Figure 3A,B, upper panels). A similar pattern was observed during ASD, although temperatures were generally lower, with daily ranges of 28.38–31.29 °C in trial 1 and 29.66–33.31 °C in trial 4. Mean temperatures for ASD trials 1–4 were 29.48, 30.02, 30.24, and 30.96 °C, respectively (Figure 3A,B, lower panels). Initial DO concentrations likewise differed among trials. During AG, trial 1 had the highest initial DO concentration (7.58 mg L^{−1}), compared with 7.14–7.28 mg L^{−1} in other trials (Figure 3C). During ASD, trial 4 had the lowest initial DO concentration (6.58 mg L^{−1}), whereas trials 1–3 ranged from 7.19 to 7.24 mg L^{−1} (Figure 3C).

The phenotypic consequences of this temperature–DO gradient were evident across both AG and ASD assays. During AG, trial 1, which combined the lowest mean ambient temperature with the highest initial DO concentration, had a higher median coleoptile length (45.67 mm) than trials 2–4 (35.96–38.49 mm; Figure 3D). The median elongation index was also higher in trial 1 (3.15) than in the other trials (2.04–2.38). Conversely, during ASD, trial 4, which combined the highest mean temperature with the lowest initial DO concentration, had a lower median coleoptile length (33.27 mm) than trials 1–3 (39.45–42.98 mm; Figure 3E). Coleoptile tissue disintegration was also more frequently observed in ASD trial 4, whereas it occurred only sporadically in the other ASD trials and rarely during AG (Figure 3D,E). Genotype-level comparisons between trials 1 and 4 likewise showed

generally reduced coleoptile elongation and more frequent tissue disintegration under the warmer, lower-DO conditions in both assays (Figure 3F,G). Together, these observations indicate that variation in ambient temperature and DO conditions was associated with trial-to-trial differences in coleoptile elongation.

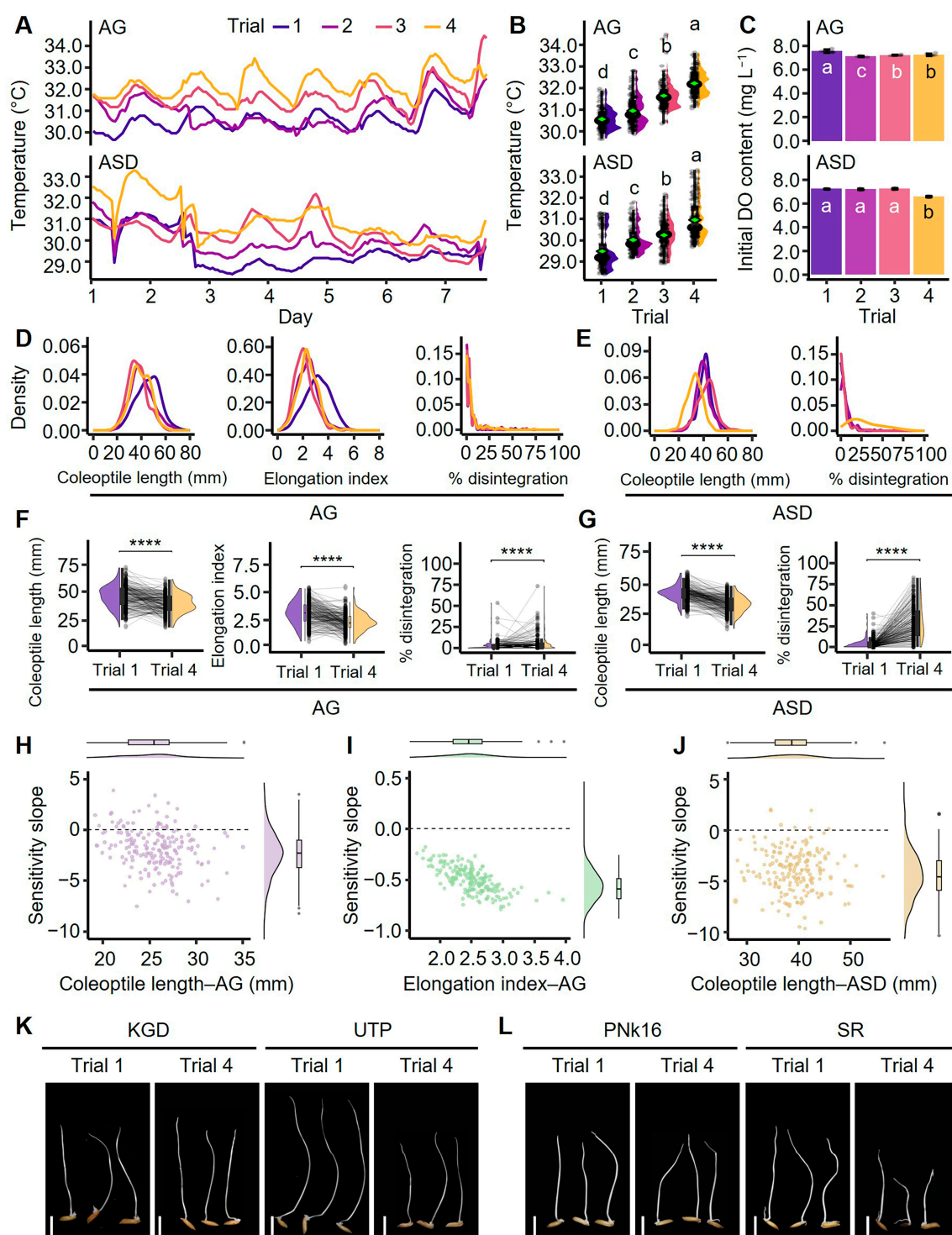


Figure 3. Trial-to-trial environmental variation and genotype-dependent coleoptile elongation responses during AG and ASD. (A) Hourly ambient temperature profiles across four trials of the AG and ASD assays. (B,C) Raincloud plots of ambient temperature (B) and bar plots of DO concentration measured before submergence (C) for each AG and ASD trial. In (B), green diamonds indicate the mean temperature across all recorded time points. In (C), data are presented as mean $\pm$ SD from

6 bottles ($n = 6$). Different letters indicate significant differences among trials according to one-way ANOVA followed by DMRT ($p < 0.05$). (D,E) Density plots showing population distributions of coleoptile length, elongation index, and coleoptile disintegration percentage across four trials during AG (D) and ASD (E). (F,G) Pairwise comparisons of the phenotypic traits shown in (D,E) between trials 1 and 4 during AG (F) and ASD (G). **** indicates $p < 0.0001$ according to the Wilcoxon signed-rank test. (H–J) Scatterplots showing the relationships between reaction-norm slopes and model-based trait means across 194 rice genotypes for coleoptile length during AG (H), elongation index during AG (I), and coleoptile length during ASD (J). Marginal density plots and boxplots summarize the corresponding distributions. Dashed horizontal lines indicate a reaction-norm slope of zero, and center lines in the boxplots indicate medians. (K) Seedlings of ‘Khao Gaw Diaw’ (KGD) and ‘U-tapao’ (UTP) from trials 1 and 4, illustrating relatively stable and environmentally sensitive coleoptile elongation responses during AG, respectively. (L) Seedlings of ‘Puang Nahk 16’ (PNk16) and ‘Sawng Ruang’ (SR) from trials 1 and 4, illustrating relatively stable and environmentally sensitive coleoptile elongation responses during ASD, respectively. Scale bars = 10 mm.

The trial means shown in Figure 1B,C and Figure 2B further indicated that the magnitude of within-genotype variation differed among accessions, suggesting genotype-dependent environmental sensitivity. We therefore asked whether accessions differed not only in their overall capacity for underwater coleoptile elongation, but also in the stability of this response across the environmental conditions encountered during screening. To quantify this variation, coleoptile length and elongation index were modelled as reaction norms across a composite environmental index, in which higher values represented warmer conditions associated with lower DO concentration. The resulting slopes were used as measures of phenotypic sensitivity across trials, rather than as estimates of a direct causal effect of temperature or DO. More negative slopes therefore indicated a greater decline in elongation across this environmental gradient. Among the 194 genotypes, reaction-norm slopes for coleoptile length during AG ranged from -7.55 to 3.88 , with a median of -1.75 (Figure 3H). Slopes for the elongation index ranged from -0.79 to -0.18 (Figure 3I), indicating that all genotypes showed some decline in this trait as the environmental index increased. During ASD, slopes for coleoptile length ranged from -9.67 to 2.02 , with a median of -4.02 (Figure 3J). The more negative distribution of slopes during ASD than during AG indicated greater sensitivity of post-germination coleoptile elongation to variation along the temperature–DO gradient.

Under both AG and ASD, some genotypes combined relatively long coleoptiles with slopes near zero, indicating comparatively stable elongation across trials, whereas others showed pronounced reductions as the environmental index increased (Figure 3K,L). Thus, the reaction-norm analysis distinguished accessions with similar overall elongation capacity but contrasting environmental sensitivity, providing information that was not captured by mean coleoptile length alone.

2.5. Elevated Temperature Lowers DO Concentration and Suppresses Coleoptile Elongation Under Submergence

Because O_2 solubility in water decreases with increasing temperature, we first characterized the relationship between water temperature and DO concentration under controlled conditions. For these experiments, we established a 50-mL tube system that allowed temperature and DO dynamics to be monitored across five temperature treatments (25, 28, 30, 32, and 34 °C). Water temperature and DO concentration were monitored at 0 and 3 h and daily for 5 days. Water temperature rapidly approached the respective set temperatures and remained relatively stable thereafter (Figure 4A). Increasing water temperature was accompanied by progressively lower DO concentrations, with differences among temperature treatments already apparent within 3 h of incubation and maintained throughout the experiment (Figure 4B).

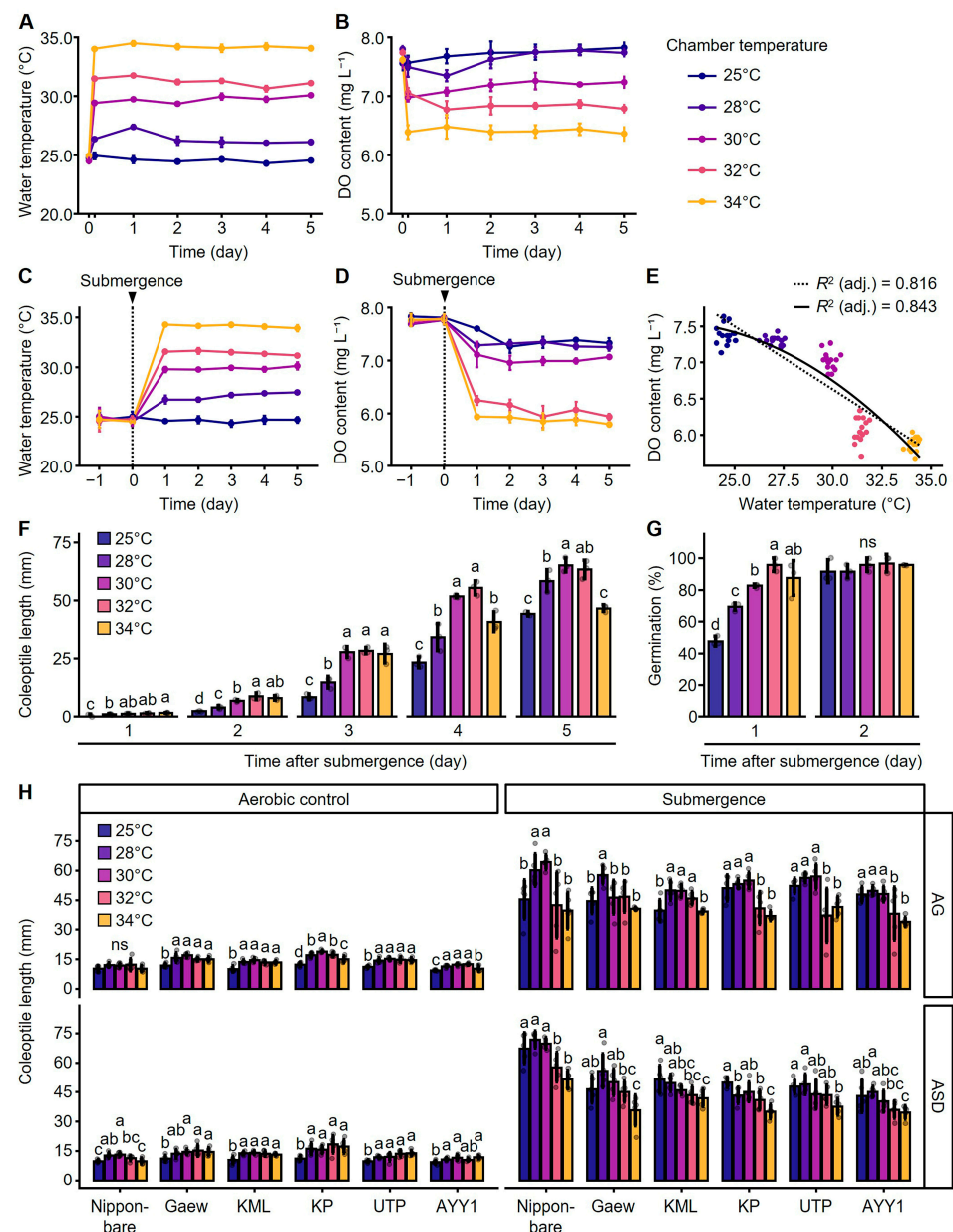


Figure 4. Water temperature affects DO dynamics and coleoptile growth during AG and ASD. (A,B) Water temperature profiles (A) and DO dynamics (B) in 50-mL tubes incubated at five set temperatures (25, 28, 30, 32, and 34 °C) without seeds. Water temperature and DO were monitored at 0 and 3 h and daily for 5 days. (C,D) Water temperature profiles (C) and DO dynamics (D) from 1 day before submergence to day 5 in tubes containing Nipponbare seeds. Water was initially equilibrated at 25 °C before seed submergence and transfer to the five temperature treatments. Data in (A–D) are presented as mean ± SD from three independent trials ($n = 3$). (E) Relationship between water temperature and DO concentration across days 1–5 after submergence, using trial-level values shown in (C,D). Dashed and solid lines denote linear and quadratic fits, respectively. (F) Coleoptile length of Nipponbare under AG conditions measured daily for 5 days after submergence at each temperature. Data are presented as mean ± SD from three independent trials ($n = 3$). (G) Germination percentage of Nipponbare at 1 and 2 days after submergence. Data are presented as mean ± SD from three independent trials ($n = 3$). (H) Coleoptile length of Nipponbare, Gaew, KML, KP, UTP, and AYY1 under aerobic control and submerged conditions during AG and ASD at each temperature. Bars represent mean ± SD from five independent trials ($n = 5$), and dots represent individual replicate values. In (F–H), different letters indicate significant differences among temperature treatments within each time point, condition, or genotype, according to one-way ANOVA followed by DMRT; ns, not significant.

We next examined DO dynamics following seed submergence. To standardize the initial conditions, water was equilibrated at 25 °C for 1 day before imbibed Nipponbare seeds were submerged and transferred to the five temperature treatments (Figure 4C). At the onset of submergence, DO concentrations were comparable among treatments, ranging from approximately 7.77 to 7.81 mg L⁻¹ (Figure 4D). After 1 day, however, DO concentrations had diverged among temperatures, reaching 6.24 and 5.93 mg L⁻¹ at 32 and 34 °C, respectively, compared with 7.11–7.60 mg L⁻¹ at 25–30 °C (Figure 4D). These differences persisted over the subsequent 5 days. Across all observations, DO concentration decreased nonlinearly with increasing water temperature, with a quadratic model providing a better fit than a linear model ($F_{1,72} = 13.95$, $p < 0.001$; $\Delta AIC = 11.3$; adjusted $R^2 = 0.843$ vs. 0.816; Figure 4E). Together, these results demonstrate a strong inverse relationship between water temperature and DO concentration under the experimental conditions.

We next examined the temporal response of coleoptile length to temperature under AG conditions (Figure 4F). At 1 day after submergence, coleoptiles remained short across all treatments, although coleoptile length at 25 °C was significantly lower than at 30–32 °C. By day 3, coleoptile length generally increased with temperature, with similar lengths observed at 30, 32, and 34 °C. Thereafter, the response diverged as coleoptile length remained high at 30–32 °C, but it became progressively lower at 34 °C relative to these temperatures. Lower temperatures (25–28 °C) also resulted in shorter coleoptiles. By day 5, coleoptile length at 34 °C was significantly lower than at 28–32 °C, indicating that prolonged exposure to the highest temperature suppressed underwater coleoptile growth. Germination was also monitored during the first 2 days of submergence (Figure 4G). After 1 day, germination was lower at 25 and 28 °C (47.62% and 69.44%, respectively) than at 30–32 °C (82.74–95.83%), but by day 2, germination exceeded 91% across all treatments with no significant differences. Thus, slower germination may partly contribute to the shorter coleoptiles observed at lower temperatures. In contrast, the reduced coleoptile length at 34 °C cannot be explained by lower germination, which did not differ significantly from that at 30–32 °C.

Based on the pronounced temperature effect observed after 5 days of submergence, we further evaluated Nipponbare together with five Thai *indica* accessions selected for long coleoptiles under both AG and ASD conditions (Figure 4H). Each assay included an aerobic control to distinguish general temperature effects from responses associated with submergence. Under aerobic conditions, coleoptiles remained relatively short, with temperature effects generally small or genotype-dependent. Submergence induced substantially greater coleoptile growth, and the temperature response differed between AG and ASD. During AG, most genotypes produced their longest coleoptiles at intermediate temperatures, whereas 34 °C generally resulted in shorter coleoptiles. In contrast, under ASD, several genotypes produced longer coleoptiles at 25–28 °C, with length generally decreasing toward 34 °C. Because ASD seeds were pre-germinated before submergence, the greater coleoptile length at 25–28 °C cannot be readily attributed to differences in germination timing. Despite variation in the magnitude and pattern of response among genotypes, reduced coleoptile length at 34 °C was a common response under submergence, supporting an inhibitory effect of elevated temperature on sustained underwater coleoptile growth.

Finally, we examined whether these temperature effects were reproduced under the larger-volume glass-bottle system and water-preparation procedure employed in the original germplasm screening, which allowed the temperature–DO response to be followed over the longer durations used for the respective AG and ASD assays (Figure S2). Following autoclaving, water equilibrated at 34 °C reached a lower DO concentration than water maintained at 30 °C (Figure S2A). In subsequent AG and ASD experiments, water was first equilibrated at 30 °C to standardize the initial DO concentration before seed submergence and transfer to 30 or 34 °C. DO remained consistently lower at 34 °C than at 30 °C

throughout submergence under both AG and ASD conditions (Figure S2B,C), accompanied by significantly shorter submerged coleoptiles at 34 °C in Nipponbare and the Thai accessions KML and UTP (Figure S2D–G). Thus, the temperature-associated reductions in DO concentration and coleoptile length were reproducible across the two experimental systems. Together, these results demonstrate the genotype-dependent temperature sensitivity of submerged coleoptile growth, with responses further influenced by the developmental stage at which submergence occurs.

2.6. Genomic Regions Associated with Coleoptile Elongation During AG and ASD

To investigate the genetic basis of underwater coleoptile elongation, we conducted GWASs for coleoptile length during AG (CL-AG), elongation index during AG (EI-AG), and coleoptile length during ASD (CL-ASD). Analyses were performed using 189 accessions with resequencing data and 106,806 high-quality exonic SNPs. For each trait, two sets of genotype-level best linear unbiased predictors (BLUPs) were generated. Design-based BLUPs accounted for trial effects and partitioned variation into genotype, genotype $\times$ trial, and residual components, whereas environment-adjusted BLUPs additionally incorporated principal components summarizing temperature profiles and initial DO concentration. Association mapping was performed using the Fixed and Random Model Circulating Probability Unification (FarmCPU) and Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway (BLINK) models. Significant markers were grouped into QTLs according to chromosome-specific linkage disequilibrium (LD) decay (Figure S3; Tables S2 and S3).

Each combination of GWAS model and phenotype adjustment detected multiple marker–trait associations and corresponding QTLs, with some variation in their number and genomic distribution among analyses (Figure 5A–F; Tables S2 and S3). To identify the most consistently supported regions, we retained only QTLs that overlapped across all four analytical combinations: FarmCPU and BLINK applied to both design-based and environment-adjusted BLUPs. The overlap among these analyses is summarized in the UpSet plots in Figure 5D–F.

For CL-AG, four QTLs were shared across all four analytical combinations (Figure 5D): *qCL-AG-1* on chromosome 1 (18.91–19.52 Mb), *qCL-AG-2* on chromosome 2 (34.72–34.92 Mb), and *qCL-AG-8.1* (5.37–5.58 Mb) and *qCL-AG-8.2* (26.86–27.09 Mb) on chromosome 8 (Figure 5G). *qCL-AG-1* overlapped a previously reported QTL for coleoptile volume, whereas *qCL-AG-8.1* and *qCL-AG-8.2* coincided with regions previously associated with anaerobic response traits [6,33–35]. No corresponding interval was identified among the previous reports examined for *qCL-AG-2*, suggesting that this association may be specific to the Thai *indica* panel.

For EI-AG, three QTLs were shared across all four analyses (Figure 5E): *qEI-AG-4* on chromosome 4 (23.84–24.12 Mb), *qEI-AG-11* on chromosome 11 (17.29–17.44 Mb), and *qEI-AG-12* on chromosome 12 (17.19–17.59 Mb) (Figure 5G). *qEI-AG-4* overlapped a reported QTL for coleoptile surface area under submergence, whereas *qEI-AG-12* coincided with previously reported regions associated with anaerobic response index and coleoptile volume [6,33,36]. No previously reported AG-associated interval was identified for *qEI-AG-11*.

For CL-ASD, one QTL, *qCL-ASD-7* on chromosome 7 (18.68–18.85 Mb), was shared across all four analytical combinations (Figure 5F,G). This region overlapped a previously reported QTL associated with flood-tolerance index [37].

Overall, four QTLs for CL-AG, three for EI-AG, and one for CL-ASD were consistently identified across both GWAS models and both phenotype-adjustment approaches (Figure 5G). Additional QTLs were detected by individual analyses but were not retained in this conservative set because they were not shared across all four combinations

(Figure 5D–F; Tables S2 and S3). The absence of overlap between the retained AG- and ASD-associated regions further supports partially distinct genetic architectures of underwater coleoptile elongation before and after germination.

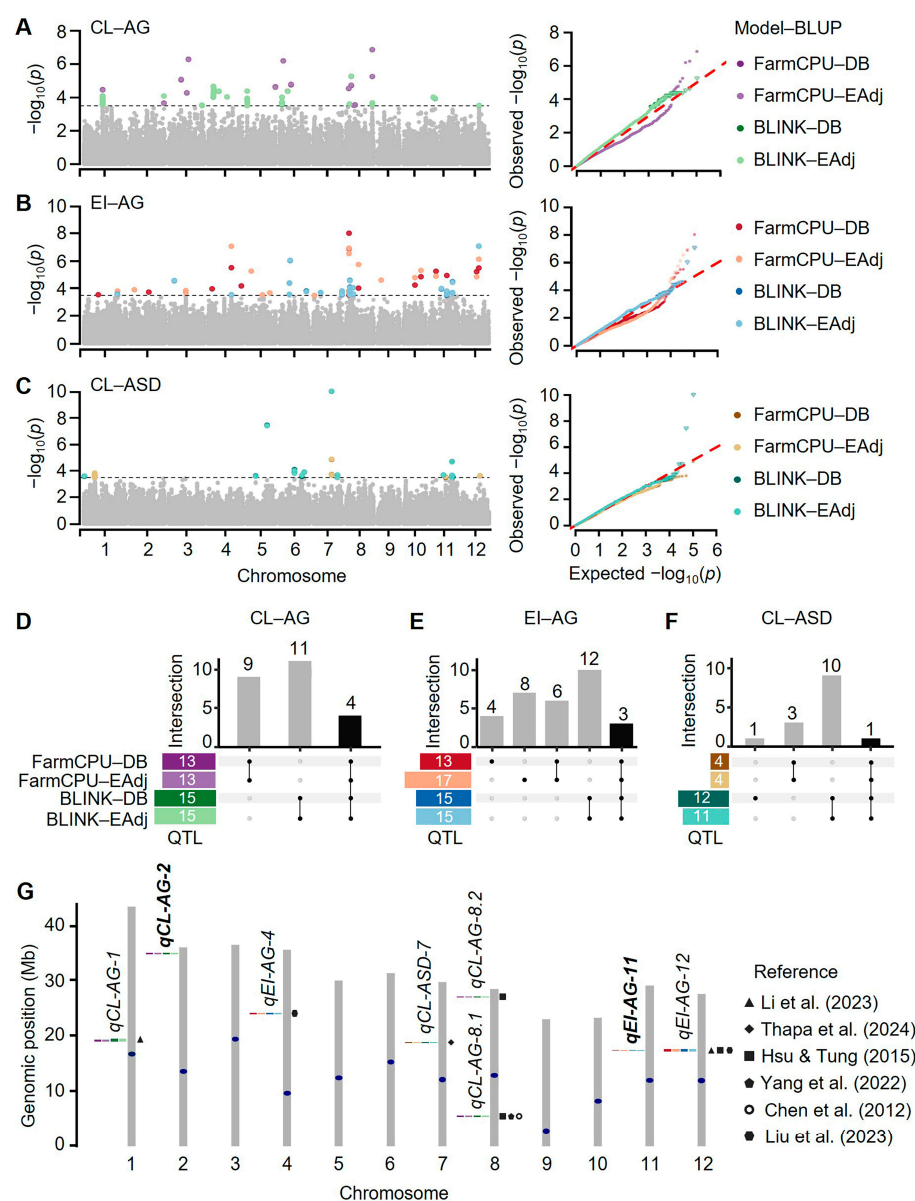


Figure 5. Genome-wide association profiles and detected QTLs for coleoptile elongation during AG and ASD. (A–C) Manhattan and quantile–quantile (Q–Q) plots for genome-wide association analyses of coleoptile length during AG (CL–AG; (A)), elongation index during AG (EI–AG; (B)), and coleoptile length during ASD (CL–ASD; (C)), using FarmCPU and BLINK models with design-based (DB) and environment-adjusted (EAdj) BLUPs. Model $\times$ BLUP combinations are color-coded. In the Manhattan plots, the x-axis indicates genomic position across chromosomes, and the y-axis indicates $-\log_{10}(p)$ for marker–trait associations. Each point represents a SNP, and the dashed horizontal line indicates the significance threshold of $-\log_{10}(p) \geq 3.5$. The Q–Q plots compare expected and observed $-\log_{10}(p)$ values across markers. (D–F) UpSet plots showing overlap among QTLs detected by FarmCPU and BLINK using DB and EAdj BLUPs for CL–AG (D), EI–AG (E), and CL–ASD (F). Connected dots identify the analytical combinations contributing to each intersection, and vertical bars indicate the number of overlapping QTLs. (G) Genomic positions of QTLs consistently detected across all four combinations of GWAS model and phenotype adjustment. Symbols to the right of each chromosome indicate previously reported AG-associated QTLs [6,33–37]. Loci not overlapping the previously reported intervals examined in this study are shown in bold.

2.7. Gene-Based Haplotype Associations Highlight Candidate Genes Within *qEI-AG-12* for Coleoptile Elongation Under Submergence

To move from association signals to putative causal genes, we prioritized *qEI-AG-12* on chromosome 12 for fine-scale dissection as it was consistently detected in all GWAS models and overlaps regions repeatedly detected for anaerobic establishment-related traits [6,33,36]. Local LD structure delimited *qEI-AG-12* to a ~410-kb interval (17.189–17.599 Mb), with the lead SNP located at the position 17,391,378 (Figure 6A). This region contained 11 annotated genes, with the lead SNP localized on *OsNAM* (*Os12g0477400*) (Table S4). To prioritize plausible candidate genes involved in coleoptile elongation under O₂ limitation, we integrated three independent RNA-seq datasets generated under AG conditions [33,38,39]. Five genes met our detectability criterion, with mean expression ≥ 5 transcripts per million (TPMs) in at least one sample across the three datasets, including *OsSWEET13* (*Os12g0476200*), *OsNAM*, *Os12g0477600*, *OsABA45* (*Os12g0478200*), and *OsARF24* (*Os12g0479400*) (Figure 6B). We then tested gene-based haplotypes for association with elongation index under AG and evaluated linkage between gene-level variants and the GWAS lead SNP.

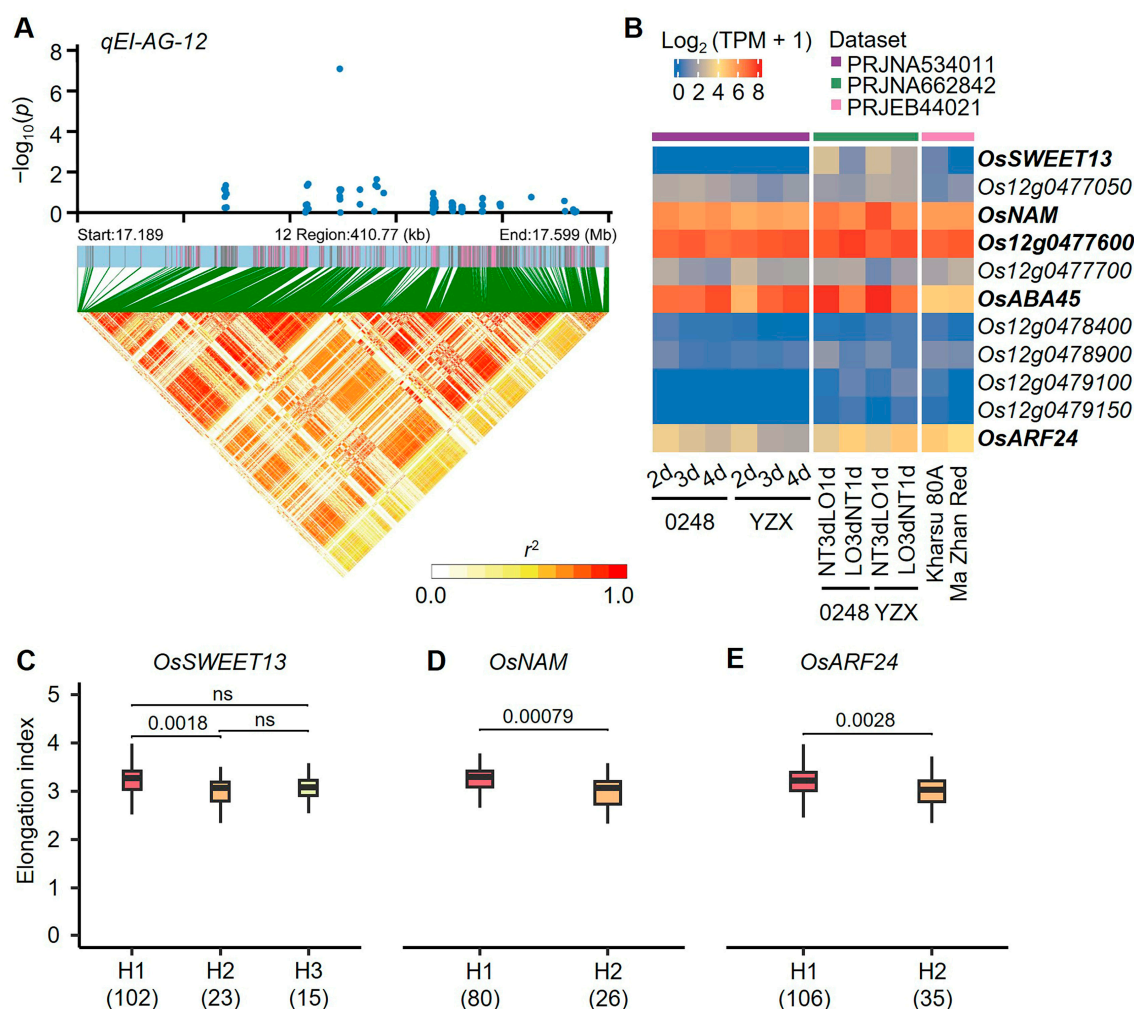


Figure 6. Haplotype analysis prioritizes candidate genes within *qEI-AG-12*. (A) Regional association (top) and local LD heatmap (bottom) spanning 17.189–17.599 Mb on chromosome 12. (B) Heatmap of transcript abundance for genes within the *qEI-AG-12* interval based on three public AG-related RNA-seq datasets. PRJNA534011 profiled whole germinating seeds of 02428 (*japonica*; tolerant) and YZX (*indica*; intolerant) under low O₂ at 2, 3, and 4 days. PRJNA662842 profiled germinating seeds of 02428 and YZX under aerobic conditions for 3 days followed by 1 day of low O₂ (NT3dLO1d), and low O₂ for 3 days followed by 1 day of aerobic conditions (LO3dNT1d). PRJEB44021 profiled coleoptile

tissue from two AG-tolerant genotypes (Kharsu 80A and Ma-Zhan Red) under low O₂ for 4 days. Within each dataset, values are shown as log₂ (TPM + 1). Genes with mean expression ≥ 5 TPM in at least one sample are highlighted in bold. (C–E) Boxplots of design-BLUP elongation index under AG across haplotype groups for *OsSWEET13* (C), *OsNAM* (D), and *OsARF24* (E). Numbers in parentheses indicate accession counts. *p*-values were calculated using two-sample *t*-tests. Haplotypes containing fewer than 10 accessions are not displayed.

Three of the five AG-expressed genes showed significant haplotype–phenotype associations, namely *OsSWEET13*, *OsNAM*, and *OsARF24* (Figure 6C–E; Tables S4 and S5). The lead SNP itself is located within *OsNAM*, while *OsSWEET13* and *OsARF24* carried 2 and 50 variants, respectively, in strong LD with the lead SNP ($r^2 \geq 0.8$). In contrast, no variants in *Os12g0477600* or *OsABA45* showed such strong LD with the lead SNP, and the haplotypes were not significantly associated with the phenotype (Table S4).

OsSWEET13, encoding a sucrose transporter, showed three major haplotypes. Its H1 haplotype ($n = 102$) showed a higher elongation index (3.24) than H2 ($n = 23$; 2.99), while H3 ($n = 15$; 3.10) did not show significant differences from either H1 or H2 (Figure 6C). *OsNAM*, encoding a no apical meristem protein, exhibited two haplotypes, with H1 ($n = 80$) showing a higher elongation index (3.27) than H2 ($n = 26$; 3.00) (Figure 6D). Finally, *OsARF24*, encoding an auxin response factor, had two haplotypes, with H1 ($n = 106$) associated with greater elongation index (3.21) than H2 ($n = 35$; 3.01) (Figure 6E).

Together, their expression under AG conditions, haplotype–phenotype associations, and LD with the GWAS lead SNP prioritize these genes as plausible candidates within *qEI-AG-12*.

3. Discussion

In this study, we characterized underwater coleoptile elongation in a diverse panel of Thai *indica* rice and identified substantial genotypic variation during both AG and ASD. Several Thai accessions produced coleoptiles comparable in length to those of Nipponbare, whereas many showed relatively weak elongation responses. Importantly, genotypic performance largely differed between submergence imposed before germination and after radicle emergence, indicating that early flooding tolerance is developmentally dependent. Screening across ambient trials further revealed pronounced environmental sensitivity, with coleoptile elongation generally reduced under warmer conditions associated with lower DO concentration. Controlled-temperature experiments supported this relationship by showing that incubation at higher temperatures lowered DO concentration and suppressed underwater elongation. GWAS provided complementary genetic evidence by identifying eight QTLs consistently detected across two phenotype-adjustment approaches and two association models.

Most studies of AG have been conducted under controlled laboratory conditions with fixed temperatures or experimentally reduced O₂ availability [5,26,28]. Such designs improve experimental precision but may not capture the environmental variation encountered during crop establishment. Greenhouse and field experiments provide greater agronomic realism and have revealed season-dependent variation in AG responses [30], but interacting factors such as temperature, solar radiation, soil O₂ demand, and water column O₂ dynamics are difficult to separate [37,40]. By conducting repeated screening under naturally varying ambient temperatures, our study captured trial-to-trial variation that would have been obscured under a single controlled environment. The resulting differences in coleoptile elongation were associated with variation in temperature and initial DO, emphasizing that apparent genotypic performance under submergence depends partly on the environment in which screening is conducted.

The comparison between AG and ASD further showed that underwater elongation responses are not fully conserved across developmental stages. Coleoptile length during AG and ASD was only weakly correlated, and several accessions showed contrasting responses between the two assays. This finding is consistent with reports that tolerance measured during germination and at later seedling stages can show weak phenotypic correlation and limited overlap in associated loci [35]. During AG, growth depends largely on the initiation of reserve mobilization and the establishment of fermentative metabolism, whereas after radicle emergence, seedlings have already entered a distinct developmental and metabolic state [41,42]. Differences in the timing of O₂ limitation may therefore alter sugar-starvation signaling, starch mobilization, hormonal regulation, and the balance between root and shoot growth. AG and ASD should consequently be regarded as related but physiologically distinct components of early submergence response rather than interchangeable measures of tolerance.

The four response categories identified from coleoptile traits under AG and ASD (Figure 2D) provide a useful framework for capturing this variation among accessions. Category 1 comprises accessions with strong responses under both conditions and therefore represents valuable germplasm for investigating traits that contribute to robust coleoptile elongation across developmental stages. Categories 2 and 3, which show preferential responses under AG and ASD, respectively, provide contrasting materials for dissecting stage-specific physiological and genetic mechanisms. These three groups also offer complementary donor resources for breeding, depending on whether tolerance across both stages or improvement at a specific stage is targeted. Although category 4 accessions showed relatively poor coleoptile performance under both conditions, this group includes agronomically important cultivars such as PTT1 and 'Niaw Khiaw Ngoo', which could serve as recipient backgrounds for introgressing favorable AG/ASD alleles while retaining desirable agronomic characteristics. Thus, rather than serving only as a descriptive classification, the four groups provide biologically contrasting germplasm for mechanistic studies and breeding applications.

Temperature emerged as an important modifier of underwater coleoptile elongation. Across ambient trials, higher temperature was associated with lower DO concentration and shorter coleoptiles, and this pattern was reproduced under controlled conditions. The reduction in DO at higher temperature is consistent with the lower solubility of O₂ in warmer water, and the relationship between water temperature and DO was better described by a quadratic than a linear model, indicating that DO did not decline at a constant rate across a temperature gradient. In addition, DO concentrations became lower after seed submergence at warmer temperatures, indicating that differences in O₂ availability persisted as seedlings developed. These effects are likely to be especially relevant during early seedling establishment, when respiratory demand increases while gas diffusion remains restricted by the surrounding water.

The contrasting temperature responses under AG and ASD further help distinguish effects on germination from those on subsequent coleoptile growth. Under AG, shorter coleoptiles at 25–28 °C coincided with slower germination during the first day of submergence, suggesting that delayed germination may partly contribute to the reduced coleoptile length at lower temperatures. This interpretation is supported by the ASD treatment, in which seeds were pre-germinated before submergence and several genotypes instead produced longer coleoptiles at 25–28 °C. Thus, the apparent disadvantage of lower temperature during AG may reflect, at least partly, its effect on the timing of germination rather than an inhibitory effect on coleoptile growth itself. In contrast, 34 °C generally reduced coleoptile length under both AG and ASD, despite no evidence of reduced germination at this temperature during AG. The persistence of this response in pre-germinated seedlings

indicates that the inhibitory effect of high temperature extends beyond germination and affects subsequent underwater coleoptile growth.

The inhibition of post-germination coleoptile growth at elevated temperature may involve several interacting processes beyond reduced O₂ solubility. Higher temperature can increase respiratory demand and accelerate consumption of available O₂ and carbohydrate reserves [43]. Under prolonged O₂ limitation, rapid carbon flux through glycolysis and fermentation may also increase the risk of substrate depletion, redox imbalance, and accumulation of potentially harmful metabolic intermediates [44]. Hormonal regulation may likewise be affected because several reactions involved in gibberellin biosynthesis require O₂ [45], while auxin, ethylene, abscisic acid, and jasmonic acid interact in controlling germination and coleoptile growth. The observed reduction in coleoptile elongation at higher temperatures may therefore result from multiple, potentially interacting processes, including reduced O₂ availability, increased metabolic demand, and temperature-sensitive regulatory responses. Importantly, the latter two may also influence coleoptile growth independently of O₂ availability. Because temperature and DO were not experimentally decoupled in the present study, their relative contributions cannot yet be distinguished. Resolving these effects will require factorial manipulation of water temperature and O₂ concentration, coupled with physiological and molecular measurements to distinguish O₂-dependent effects from direct temperature responses.

Genotypes also differed in the stability of coleoptile elongation across ambient trials. Reaction-norm analysis identified accessions that maintained relatively long coleoptiles across the temperature–DO gradient, whereas others showed steep declines under warmer, lower DO conditions. This variation suggests that rice germplasm differs not only in maximum elongation capacity but also in environmental sensitivity. Such differences may arise from variation in reserve mobilization, respiratory efficiency, fermentation capacity, membrane stability, antioxidant protection, or hormonal responsiveness [14]. Accessions combining strong elongation with relatively stable reaction-norms may therefore be useful for investigating mechanisms that buffer early seedling growth against fluctuating submergence environments.

The variance-component analysis further indicated that coleoptile length contained substantial genotypic variation under both AG and ASD, although environmental contributions were greater during AG. The comparatively wider PCV–GCV difference and lower heritability of the elongation index also indicate that this derived trait was more sensitive to trial conditions than absolute coleoptile length. By contrast, germination percentage varied relatively little across the panel despite moderate-to-high heritability. These findings support the use of coleoptile elongation as an informative quantitative trait for characterizing early submergence responses, while also showing that replicated screening is necessary when phenotyping is performed under variable environments.

GWAS was used as a complementary approach to determine whether the observed phenotypic variation was associated with reproducible genomic regions. By retaining only QTLs recovered across FarmCPU and BLINK using both design-based and environment-adjusted BLUPs, we identified four QTLs for CL-AG, three for EI-AG, and one for CL-ASD. Several coincided with previously reported regions associated with anaerobic response, germinability, coleoptile surface area, coleoptile volume, or flood-tolerance indices [6,33–37], supporting the biological relevance of the detected associations. The recovery of *qCL-AG-2* and *qEI-AG-11* outside the previously reported intervals further suggests that Thai *indica* germplasm contains additional genetic variation affecting submerged coleoptile elongation. Notably, the retained AG- and ASD-associated loci did not overlap, consistent with the weak phenotypic correlation between the two assays and further supporting partially distinct genetic architectures before and after germination.

Among the candidates at *qEI-AG-12*, *OsSWEET13* and *OsARF24* have the strongest prior biological support for roles in submerged coleoptile elongation. *OsSWEET13* encodes a sucrose transporter and belongs to a group of *SWEET* genes whose expression in submerged coleoptiles is linked to auxin-dependent elongation [13]. *OsARF24* encodes an auxin response factor [46], consistent with the central role of auxin signaling in promoting cell elongation under O₂ limitation [13,21]. Although *OsARF24* expression decreases after prolonged anaerobic treatment [47], its regulation during the earlier phase of coleoptile elongation remains unclear. Together with their haplotype associations and strong LD with the lead SNP, these functional links support *OsSWEET13* and *OsARF24* as plausible candidates connecting carbohydrate mobilization and auxin signaling to variation in AG response.

OsNAM, in contrast, is particularly compelling from a genetic perspective because it contains the GWAS lead SNP and its haplotypes are associated with elongation index. However, its characterized functions are primarily related to drought tolerance and root development [48], with no established role in AG or coleoptile elongation. Thus, *qEI-AG-12* may reflect a causal variant within *OsNAM*, linked variation affecting *OsSWEET13* or *OsARF24*, or contributions from multiple alleles across the locus. Distinguishing these possibilities will require functional validation of the candidate genes and associated variants under AG conditions.

Overall, our results show that underwater coleoptile elongation is shaped by interactions among genotype, developmental stage, and environmental conditions. Thai *indica* rice contains broad variation in elongation responses during both AG and ASD, but performance in one stage does not necessarily predict performance in the other. Elevated temperature suppresses underwater elongation and coincides with lower DO content, highlighting the need to consider temperature when evaluating early submergence responses. Integrating repeated phenotyping, controlled environmental experiments, reaction-norm analysis, and GWAS provides a framework for identifying both stable genotypes and biological processes that contribute to rice establishment under variable flooding conditions.

4. Materials and Methods

4.1. Plant Materials

The germplasm comprised 192 Thai rice accessions and two reference varieties, Nipponbare and IR64. The Thai accessions represent a previously characterized local *indica* diversity panel [49], with their provinces of origin provided in Table S1. Nipponbare (*japonica*) and IR64 (*indica*) were included as reference varieties because of their contrasting responses to AG, representing AG-tolerant and AG-sensitive phenotypes, respectively [6]. Seeds of all genotypes were harvested during the same growing season, sun-dried, and stored at 4 °C at the Center of Excellence in Environment and Plant Physiology, Chulalongkorn University, until use. Prior to each experiment, seeds were manually selected to ensure that only fully developed, visually healthy, and undamaged seeds were used for phenotyping.

4.2. Phenotyping of Coleoptile Elongation Under Natural Ambient Environmental Conditions

Coleoptile elongation was evaluated under submergence imposed either before germination or after radicle emergence, representing AG and ASD, respectively. Phenotyping was conducted under ambient environmental conditions using a completely randomized design with four independent trials, each containing 40 seeds per genotype. Seeds stored at 4 °C were equilibrated to room temperature for 2 days, surface-sterilized with 10% (*v/v*) Clorox® for 10 min, rinsed 7–8 times with sterile reverse-osmosis (RO) water, and imbibed in sterile RO water for 16 h.

AG assays were conducted between March and June 2024. After imbibition, seeds were transferred into glass bottles containing a 10-cm column of sterile RO water (640 mL). Bottles were maintained in complete darkness for 7 days to allow germination and seedling growth. In parallel, an aerobic control was established in darkness by placing imbibed seeds on a floating plastic frame in Petri dishes containing RO water at a depth of 1 cm for 7 days.

ASD assays were conducted between July and September 2024. After imbibition, seeds were incubated under a wet cloth in darkness for 30 h to allow radicle emergence and were then transferred into glass bottles containing a 10-cm column of sterile RO water. Seedling development continued under submergence in darkness for 5 days. A separate aerobic control was not included in the ASD assay because coleoptile length under aerobic conditions showed limited genotypic variation in the AG experiment.

For both AG and ASD assays, RO water was autoclaved, and the bottles were maintained under naturally varying ambient temperatures for 4 days to allow water temperature and DO to equilibrate before submergence. DO concentration was measured immediately before submergence using a U26 sensor (Onset HOBO, Bourne, MA, USA), and ambient air temperature was recorded hourly using a pendant temperature/light sensor (Onset HOBO, USA). At the end of each assay, seedlings were photographed, and coleoptile length was measured from the seed base to the coleoptile tip by using ImageJ (version 1.54g). Coleoptile tissue integrity was also visually assessed for each seedling. Coleoptiles were classified as disintegrated when they showed clear tissue degradation and loss of structural integrity, ranging from partial degradation to complete loss of the coleoptile, with only a short remnant remaining attached to the seed. These symptoms were readily distinguished from the intact coleoptiles of normally developed seedlings. For each genotype and trial, coleoptile disintegration is expressed as the percentage of seedlings showing these symptoms relative to the total number of seeds evaluated.

4.3. Determination of Coleoptile Elongation Under Controlled Temperatures

Controlled-temperature experiments were conducted using two complementary assay systems. A small-scale 50-mL tube system was used to characterize water temperature and DO dynamics and coleoptile responses across a five-temperature gradient, whereas the glass-bottle system described in Section 4.2 was subsequently used to confirm the temperature response under the conditions employed for germplasm screening.

For the 50-mL tube system, water temperature and DO dynamics were first characterized at five set temperatures (25, 28, 30, 32, and 34 °C). Tubes containing an 8-cm column of non-autoclaved RO water without seeds were incubated at each temperature, and water temperature and DO concentration were monitored at 0 and 3 h and daily for 5 days. To examine DO dynamics following seed submergence, RO water was first equilibrated at 25 °C for 1 day to standardize the initial conditions. Imbibed Nipponbare seeds were then submerged in the tubes (8 seeds per tube), which were transferred to the five temperature treatments and maintained in darkness for 5 days. Water temperature and DO concentration were monitored daily throughout the experiment. Three independent trials were performed.

The effect of temperature on coleoptile growth during AG was evaluated concurrently in Nipponbare using the same 50-mL tube system. Coleoptile length was measured daily for 5 days after submergence, and germination percentage was recorded during the first 2 days. Based on the temperature responses observed in Nipponbare, the experiment was extended to five Thai *indica* accessions (Gaew, KML, KP, UTP, and AYY1), together with Nipponbare, under AG, ASD, and aerobic control conditions across the same five-temperature gradient. For AG, imbibed seeds were submerged and incubated in darkness for 5 days at each

temperature. For ASD, imbibed seeds were pre-germinated under a wet cloth for 30 h to allow radicle emergence before submergence and incubation for 5 days at each temperature. For aerobic controls, imbibed seeds were placed on floating plastic frames in Petri dishes and incubated in darkness for 5 days at the corresponding temperatures. Five independent trials were performed for each genotype, condition, and temperature combination.

To confirm the temperature response using the glass-bottle system employed for the original germplasm screening, controlled experiments were conducted at 30 and 34 °C with Nipponbare and the Thai *indica* accessions KML and UTP. Unless otherwise specified, seed preparation, water depth and volume, and AG and ASD procedures followed those described in Section 4.2. Glass bottles containing a 10-cm column (640 mL) of autoclaved RO water were initially maintained at 30 °C for 4 days to allow water temperature and DO concentration to equilibrate. The effect of temperature on DO equilibration following autoclaving was also determined by maintaining bottles without seeds at either 30 or 34 °C. For experiments with seeds, bottles were initially equilibrated at 30 °C to standardize DO conditions before submergence and were then either maintained at 30 °C or transferred to 34 °C.

For AG, imbibed seeds were submerged and incubated in darkness for 7 days at 30 or 34 °C. For ASD, imbibed seeds were pre-germinated at 30 °C for 30 h to allow radicle emergence and were then submerged for 5 days at 30 or 34 °C. Aerobic controls were conducted using floating plastic frames in Petri dishes containing RO water at a depth of 1 cm and incubated in darkness at the corresponding temperatures. Each treatment comprised 40 seeds per replicate, with five biological replicates. Water temperature and DO concentration were measured daily using a DO-5512SD meter (Lutron, Taipei, Taiwan), while chamber air temperature was recorded hourly. At the end of each treatment, seedlings were photographed, and coleoptile length was measured using ImageJ as described in Section 4.2.

4.4. Statistical and Phenotypic Data Analysis

All statistical analyses were conducted in R version 4.4.3. Mean comparisons among three or more groups were performed using one-way ANOVA, followed by DMRT implemented in the agricolae package [50]. Pairwise comparisons were performed using paired- or independent-samples *t*-tests, or the corresponding Wilcoxon signed-rank or rank-sum tests, depending on the experimental design and distributional assumptions. Statistical significance was defined as $p < 0.05$.

The relationship between water temperature and DO concentration in the controlled-temperature experiment was evaluated using trial-level observations pooled across days 1–5 after submergence ($n = 75$; five temperatures $\times$ three independent trials $\times$ five days). Linear and quadratic regression models were fitted with DO concentration as the response variable and water temperature as the predictor. Model fits were compared using a nested *F*-test, adjusted R^2 , and Akaike Information Criterion (AIC). Homoscedasticity and normality of residuals from the quadratic model were assessed using the Breusch–Pagan and Shapiro–Wilk tests, respectively. Graphs were generated using the ggplot2 package [51].

For each AG trial, the coleoptile elongation response was summarized using an elongation index (EI):

$$EI_{ij} = \frac{\bar{L}_{\text{sub},ij} - \bar{L}_{\text{aer},ij}}{\bar{L}_{\text{aer},ij}}, \quad (1)$$

where $\bar{L}_{\text{sub},ij}$ and $\bar{L}_{\text{aer},ij}$ are the mean coleoptile lengths under submerged and aerobic conditions, respectively, for genotype *i* in trial *j*. EI was not calculated for ASD because a corresponding aerobic control was not included in the ASD assay. Genotypes were clustered according to coleoptile length and EI during AG and coleoptile length during

ASD using the PAM algorithm implemented in the cluster package [52]. Cluster profiles were visualized using ComplexHeatmap [53].

4.5. Environmental Index and Reaction-Norm Analysis

To characterize genotypic responses to environmental variation among trials, a composite environmental index was constructed from mean natural ambient temperature during each assay and mean initial DO concentration. Both variables were standardized as z-scores. The standardized DO value was multiplied by -1 so that a lower DO concentration corresponded to a higher index value. For trial j , the environmental index was calculated as the mean of the standardized temperature and inverted DO values. Higher index values therefore represented relatively warmer conditions associated with lower initial DO concentration.

Coleoptile length during AG and ASD was analyzed at the individual-seedling level using a linear mixed model containing genotype-specific random intercepts and slopes for the environmental index, following a reaction-norm approach [54]. For coleoptile length y_{ijk} of seedling k of genotype i in trial j , the model was

$$y_{ijk} = \mu + \beta E_j + (u_{0i} + u_{1i}E_j) + e_{ijk}, \quad (2)$$

where μ and β are the fixed intercept and slope, E_j is the environmental index for trial j , u_{0i} and u_{1i} are the random intercept and slope for genotype i , and e_{ijk} is the residual error. EI during AG was analyzed analogously using genotype $\times$ trial means. Models were fitted by restricted maximum likelihood (REML) using lmer in the lme4 package [55].

For genotype i , the reaction-norm slope was calculated as

$$\text{slope}_i = \beta + u_{1i}, \quad (3)$$

and the model-based trait value under the average observed environment ($E_j = 0$) was calculated as

$$\text{mean}_i = \mu + u_{0i} \quad (4)$$

Slopes close to zero indicated relatively stable trait expression across the observed environmental gradient, whereas slopes with larger absolute values indicated greater environmental responsiveness. Negative slopes represented declining trait values as temperature increased and initial DO content decreased.

4.6. Estimation of Genetic Parameters

Broad-sense heritability on a genotype-mean basis (H^2) and related genetic parameters were estimated from linear mixed models fitted by REML using lmer. Genotype was treated as a random effect and trial as a fixed effect.

For coleoptile length measured on individual seedlings, the model was

$$y_{ijk} = \mu + t_j + g_i + (gt)_{ij} + e_{ijk}, \quad (5)$$

where y_{ijk} is the coleoptile length of seedling k from genotype i in trial j , μ is the overall mean, t_j is the fixed effect of trial j , g_i is the random effect of genotype i , $(gt)_{ij}$ is the random genotype $\times$ trial interaction, and e_{ijk} is the residual error.

EI and germination percentage were available as genotype $\times$ trial summaries and were analyzed using

$$y_{ij} = \mu + t_j + g_i + e_{ij}, \quad (6)$$

where e_{ij} is the residual error among genotype $\times$ trial summaries. In both models (5) and (6), σ_g^2 denotes the genotypic variance (variance among genotype effects), and σ_e^2 denotes the residual variance. For individual-seedling data, the phenotypic variance of genotype means (σ_p^2) was calculated as

$$\sigma_p^2 = \sigma_g^2 + \frac{\sigma_{gt}^2}{t} + \frac{\sigma_e^2}{t\bar{n}}, \quad (7)$$

where t is the number of trials, σ_{gt}^2 denotes genotype $\times$ trial variance, and $\bar{n}$ is the harmonic mean number of seedlings per genotype per trial. For genotype $\times$ trial summaries, phenotypic variance was calculated as

$$\sigma_p^2 = \sigma_g^2 + \frac{\sigma_e^2}{t} \quad (8)$$

Broad-sense heritability on a genotype-mean basis was then calculated as

$$H^2 = \frac{\sigma_g^2}{\sigma_p^2}, \quad (9)$$

following the variance-component framework for entry-mean heritability [56]. Genotypic and phenotypic coefficients of variation (GCV and PCV) were calculated according to Singh and Chaudhary [57]. Expected genetic advance under a selection intensity of 5% and genetic advance as a percentage of the mean (GAM) were calculated according to Johnson et al. [58].

4.7. Phenotypes for Genome-Wide Association Analysis

4.7.1. Design-Based BLUPs

Design-based phenotypes for GWAS were obtained as genotype-level BLUPs from the mixed models used for heritability estimation. For coleoptile length, the predicted random genotype effect $\hat{g}_i$ from model (5) was added to the intercept $\hat{\mu}$ to obtain a trial-adjusted genotype value $\hat{\mu} + \hat{g}_i$. The same procedure was applied to EI using model (6). These values accounted for trial effects and shrinkage associated with the precision of each genotype estimate.

4.7.2. Environment-Adjusted BLUPs

A second phenotype-adjustment approach was used to account additionally for measured environmental variation among trials. For each trial, time-series data of ambient temperature and initial DO were summarized into a reduced set of trial-level environmental covariates by using principal component analysis (PCA). The first two PCs of temperature and DO (temp_pc1, temp_pc2, do_pc1, do_pc2) were calculated from standardized time-series and mean-centered. These trial-level covariates were matched to genotype $\times$ trial trait values. Genotype $\times$ trial phenotypes y_{ij} were analyzed using the following equation:

$$y_{ij} = \mu + \beta_1 \text{temp_pc1}_j + \beta_2 \text{temp_pc2}_j + \beta_3 \text{do_pc1}_j + \beta_4 \text{do_pc2}_j + g_i + e_{ij}, \quad (10)$$

where μ is the intercept, β_1 – β_4 are fixed regression coefficients for the environmental PCs, g_i is the random effect of genotype i , and e_{ij} is the residual error. Models were fitted by REML using lmer. Environment-adjusted genotype means were calculated as $\hat{\mu} + \hat{g}_i^{(\text{env})}$. The values represent genotype performance after adjustment for the included temperature and DO covariates.

4.8. Genotype Data and SNP Filtering

SNP genotype data for the Thai rice diversity panel were obtained from the previously published dataset [49]. Briefly, genomic DNA was extracted from young leaf tissue using the Genomic DNA Mini Kit (Plant; Geneaid Biotech Ltd., Taipei, Taiwan) according to the manufacturer's protocol. DNA quantity and integrity were assessed using a NanoDrop™ spectrophotometer and agarose gel electrophoresis, respectively. Genomic DNA was sequenced using an Illumina Genome Analyzer, and the resulting reads were mapped to the rice reference genome IRGSP-1.0 using BWA (version 0.7.1). Variants were called using GATK (version 4.1) and filtered according to the sequencing-depth and genotype-quality criteria described in [49]. For the present analysis, genotype data were obtained from the published variant call format (VCF) file, and variants segregating among the 189 accessions included in this study were retained. SNPs with a minor allele frequency < 0.05 or missing genotype calls were removed, resulting in 1,017,836 high-quality genome-wide SNPs for subsequent genetic analyses.

For GWASs, the marker set was further restricted to exonic SNPs to focus on variants more likely to affect gene function. Genome-wide SNP positions were intersected with exon coordinates from the Ensembl Plants GFF3 annotation for *Oryza sativa* release 60 using BEDTools (version 2.31.1), resulting in 106,806 exonic SNPs. This marker set was used for association mapping, construction of SNP-derived kinship matrices, and estimation of marker-based heritability.

4.9. Linkage Disequilibrium Analysis

Linkage disequilibrium (LD) decay was characterized separately for each chromosome using 1,017,836 genome-wide SNPs. Chromosome-specific VCF files were analyzed using PopLDdecay version 3.43 [59] with default settings to compute pairwise LD (r^2). Mean r^2 was summarized across physical-distance bins beginning at 2 kb and increasing in 20-kb intervals. For each chromosome, the LD-decay distance was defined as the physical distance at which mean r^2 declined to 0.2. The resulting chromosome-specific distances were used as the windows for grouping nearby significant SNPs into QTL regions in GWAS post-processing.

4.10. Genome-Wide Association Mapping

GWAS analyses were performed in R using GAPIT version 3 [60]. For each trait, separate analyses were conducted using (i) design-based and (ii) environment-adjusted BLUPs as phenotypes, together with the 106,806 exonic SNPs. Two multi-locus association models, FarmCPU [61] and BLINK [62], were applied.

To account for population structure and relatedness, the first two PCs from genotype-based PCA were included as fixed covariates (PCA.total = 2). A marker-based kinship matrix was calculated in GAPIT using the centered identity-by-state (IBS) method (kinship.algorithm = "Zhang").

Marker-based heritability (MBH) was estimated using the genomic mixed model implemented in GAPIT, in which phenotypic variance was partitioned into additive genetic (σ_g^2) and residual variance (σ_e^2). MBH was calculated as

$$MBH = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2} \quad (11)$$

Following Kaler and Purcell [63], the trait-specific significance thresholds were derived from MBH using the empirical relationship for rice:

$$y = 2.53 + 0.017x, \quad (12)$$

where y is the suggested $-\log_{10}(p)$ threshold and x is MBH expressed as a percentage. Because the resulting thresholds differed slightly among traits, the highest MBH-derived threshold ($-\log_{10}(p) \geq 3.5$) was applied uniformly across all traits, phenotype-adjustment approaches, and GWAS models.

Q-Q plots of expected and observed $-\log_{10}(p)$ values were used to assess model performance and the adequacy of population structure and relatedness correction. Manhattan and Q-Q plots were generated using the CMplot package [64]. Significant SNPs located within the chromosome-specific LD-decay distance were grouped into a single QTL region. Overlap among QTLs detected across GWAS models and phenotype-adjustment approaches was summarized using ComplexUpset [65], and chromosomal positions of candidate QTLs were visualized using shinyChromosome [66].

4.11. LD-Based QTL Definition, Candidate Genes, and Haplotype Analysis

Significant SNPs were grouped into QTLs based on the chromosome-specific LD-decay distances. SNPs located within one LD-decay distance (mean $r^2 = 0.2$) of each other were merged into a single QTL. Local LD structure and haplotype blocks surrounding significant SNPs were visualized using LDBlockShow [67].

SNPs within each QTL were annotated using the Ensembl Variant Effect Predictor (VEP) for *Oryza sativa* [68]. Genes within QTL intervals were further annotated with gene models and functional descriptions from the Rice Annotation Project Database (RAP-DB). To evaluate expression support for candidate genes, we re-analyzed RNA-seq datasets from NCBI SRA BioProjects PRJNA534011 [38], PRJEB44021 [39], and PRJNA662842 [33]. Raw reads were quality-checked with FastQC version 0.12.1 [69], and trimmed using Trimmomatic version 0.39 [70] with the parameter TruSeq3-PE-2.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:30 AVGQUAL:30 MINLEN:50. Filtered reads were quantified against the rice reference transcriptome (Ensembl Plants release 62, cDNA) using Salmon version 1.10.2 with default settings [71]. Gene-level TPM values were obtained, and genes with mean expression ≥ 5 TPM in at least one sample across the three datasets were considered detectably expressed under AG conditions. For gene-based haplotype analysis, variants located within gene bodies and 5-kb upstream promoter regions were extracted from the 1,017,836-SNP genome-wide dataset using gene ID following VEP annotation. Gene-based haplotype analysis was conducted, and associations between gene haplotypes and phenotypes were tested using the geneHapR package [72].

5. Conclusions

Underwater coleoptile elongation in Thai *indica* rice is shaped by the combined effects of genotype, developmental stage, and environment. Responses during AG and ASD were only weakly correlated, showing that tolerance to submergence before and after germination represents partly distinct developmental responses. Repeated screening under natural ambient environmental conditions revealed substantial genotype-dependent variation in environmental sensitivity, while controlled-temperature experiments demonstrated that elevated temperature reduced coleoptile elongation and coincided with lower DO concentration. Several Thai accessions maintained strong elongation across both assays or showed comparatively stable responses across trials, providing useful material for physiological investigation and improvement of rice establishment under flood-prone conditions. Complementary GWAS identified eight QTLs consistently recovered across two phenotype-adjustment approaches and two association models, supporting a genetic basis for the observed variation. Six QTLs colocalized with previously reported coleoptile-related loci, while two (*qCL-AG-2* and *qEI-AG-11*) did not overlap with previously reported loci and may represent novel regions associated with underwater coleoptile elongation. Within the

recurrent chromosome 12 locus *qEI-AG-12*, LD-based refinement and integrative filtering highlighted three potential candidates (*OsSWEET13*, *OsNAM*, and *OsARF24*) for future functional validation. Together, these findings emphasize the need to consider developmental timing and temperature–O₂ dynamics when evaluating early submergence responses in rice.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/stresses6040073/s1>, Table S1: Information of Thai rice cultivars used in this study; Table S2: GWAS significant SNPs identified by FarmCPU and BLINK for coleoptile elongation using either design-based or environment-adjusted BLUPs; Table S3: LD-based QTLs for coleoptile elongation detected by FarmCPU and BLINK using design-based and environment-adjusted BLUPs; Table S4: List of genes within *qEI-AG-12*; Table S5: Haplotypes and sequences of three candidate genes in *qEI-AG-12*; Figure S1: Seed germination under air control, AG, and ASD conditions; Figure S2: Effects of elevated temperature on DO dynamics and coleoptile growth during AG and ASD in the glass-bottle screening system; Figure S3: Linkage disequilibrium (LD) analysis across 12 chromosomes in 189 Thai rice accessions.

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Abbreviations

The following abbreviations are used in this manuscript:

AG	Anaerobic germination
ASD	Anaerobic seedling development
BLINK	Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway
BLUP	Best linear unbiased prediction
CL-AG	Coleoptile length under AG
CL-ASD	Coleoptile length under ASD
DO	Dissolved oxygen
DSR	Direct-seeded rice

EI-AG	Elongation index under AG
FarmCPU	Fixed and random model Circulating Probability Unification
GWAS	Genome-wide association study
LD	Linkage disequilibrium
MBH	Marker-based heritability
O ₂	Oxygen
QTL	Quantitative trait locus
SNP	Single-nucleotide polymorphism

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