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Identification of a Novel QTL for Chlorate Resistance in Rice (*Oryza sativa* L.)

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Abstract: Chlorate resistance analysis is an effective approach commonly used to distinguish the genetic variation between *Oryza sativa* L. ssp. *indica* and *japonica*, and predict the nitrogen use efficiency (NUE). This study aimed at investigating the response of a doubled haploid (DH) population derived from anther culture of 93-11 × Milyang352 exposed to 0.1% potassium chlorate ($KClO_3$) at the seedling stage. The results revealed that the parental rice lines 93-11 (*indica*) and Milyang352 (*japonica*) showed distinctive phenotypic responses. The parental line 93-11 scored highly sensitive (0% survival) and Milyang352 scored resistant (66.7% survival) 7 days after treatment. The DH lines reflected the differential phenotypic response observed in parental lines. Interestingly, we identified a novel quantitative trait locus (QTL) for chlorate resistance on chromosome 3 (*qCHR-3*, 136 cM, logarithm of the odds—LOD: 4.1) using Kompetitive Allele-Specific PCR (KASP) markers. The additive effect (−11.97) and phenotypic variation explained (PVE; 14.9%) indicated that the allele from Milyang352 explained the observed phenotypic variation. In addition, shoot growth showed a significant difference between parental lines, but not root growth. Moreover, in silico analysis identified candidate genes with diverse and interesting molecular and physiological functions. Therefore, this study suggested that the QTL *qCHR-3* harbors promising candidate genes that could play a role in the regulation of nitrogen metabolism in rice.

Keywords: quantitative trait locus; chlorate resistance; nitrogen use efficiency; doubled haploid; rice

1. Introduction

Rice is the only cereal crop solely cultivated for human consumption, in addition to being the staple food crop for more than half of the world's population [1]. Among the cultivated species, *Oryza sativa* L. is the most cultivated species across the globe [2], and comprises two subspecies, *indica* and *japonica*. The *indica* and *japonica* rice are reported to have a large genetic variation [3,4], which could be explained in part by the evolutionary genetic differentiation and the independent domestication processes of both rice subspecies investigated through comparative genomic studies [5].

To distinguish the genetic variation between *indica* and *japonica* rice subspecies, the resistance to potassium chlorate ($KClO_3$) is commonly used as a reliable strategy, and has been shown to be effective [6,7]. Genotypes exhibiting a high degree of chlorate resistance are expected to have low nitrogen use efficiency (NUE), while those showing a sensitive phenotypic response toward potassium chlorate ($KClO_3$) are expected to have high NUE [8]. A recent report supported that the *indica* and *japonica* subspecies differ in their ability to assimilate nitrate (NO_3), and therefore have a differential nitrogen use efficiency (NUE) [8]. Gao and colleagues [9] identified nitrate reductase (*OsNR2*) as the

major component explaining NUE difference, and the *indica* allele of *NR2* confers high NUE. The *indica* allele of nitrate reductase (*OsNR2*) was reported to promote NO_3 uptake, while interacting with the nitrate transporter, *OsNRT1.1B*, resulting in improved NUE [10]. The *japonica* group is typically known for having a higher degree of resistance to $KClO_3$ compared with the *indica* type [11].

In a converse approach, $KClO_3$ is also used to investigate nitrogen metabolism in higher plants, by monitoring nitrate (NO_3) uptake, transport, and assimilation, as the main source of nitrogen [12]. In addition, chlorate (ClO_3) and nitrate (NO_3) are substrates for the nitrate reductase (NR) enzyme that reduces ClO_3 to the toxic chlorite, inducing high toxicity in plants [13–15]. Chlorates have been reported to have an inhibitory effect on the reducing activity of NR [13]. A number of studies have used ClO_3 to isolate mutant plants that are defective in nitrate reduction [14,16], while others supported that when ClO_3 was applied exogenously to *Arabidopsis* plants, an increase in NR mRNA level was observed, but the total NR protein synthesis was not affected [16].

The rate-limiting step of the NO_3 assimilation pathway has been suggested to be involved in the reduction of NO_3 to nitrite (NO_2), which is catalyzed by nitrate reductase [12]. Furthermore, chlorate has been shown to play an important role as a selective inhibitor in dissimilatory nitrate reduction, which is part of the nitrogen biological cycle [17].

Potassium chlorate ($KClO_3$) belongs to the group of chlorates known as oxidizers having a strong toxic effect on plants when applied for a long time or at a high dose [18,19]; they are able to induce oxidative stress, which may result in oxidative damage and lipid peroxidation [20].

The primary step of nitrogen acquisition by roots is the active transport across the plasma membrane of root epidermal and cortical cells. In higher plants, various nitrate transporters/ peptide transporter (NRT1/PTR) and ammonium (NH_4) transporters (AMTs) are identified, and reported to be involved in nitrogen uptake and assimilation [21]. Nitrogen assimilation is the formation of organic nitrogen compounds like amino acids from inorganic nitrogen compounds present in the environment. Organisms like fungi, certain bacteria and the majority of plants that cannot fix nitrogen gas (N_2) depend on the ability to assimilate nitrate or ammonia for their needs. Other organisms, such as animals, depend entirely on organic nitrogen for their food [22].

Recent advances in plant molecular breeding and genomics have revolutionized the understanding of the genetic response mechanisms of plants to diverse environmental cues. The application of strategic approaches, such as Genome-Wide Association Studies (GWAS) [23–25] and OMICS [26], and the emergence of genome sequencing technologies [27,28] significantly increased in the last two decades with the purpose of investigating the functional genetic components of plants involved in the control of major quantitative traits in plants. Genetic mapping and linkage analysis, coupled with GWAS, are considered as powerful tools that help to identify genetic loci affecting important traits in plants, including rice as the established model plant for monocots [29–31], and predict quantitative trait loci (QTLs) [32] contributing to the overall plant mechanism under various conditions.

Thus, this study aimed at identifying QTLs associated with chlorate resistance in rice using Kompetitive Allele-Specific PCR (KASP) markers. Therefore, a doubled haploid rice population and parental lines were exposed to potassium chlorate ($KClO_3$) at the seedling stage. The linkage groups were constructed and QTLs were analyzed for all traits considered.

2. Materials and Methods

2.1. Mapping Population and Potassium Chlorate Treatment

The mapping population was a doubled haploid (DH) population derived from anther culture of the cross between 93-11 and Milyang352 (typical *indica* subspecies (P1) and *japonica* subspecies (P2), respectively) [33]. A total of 117 rice doubled haploid lines developed through anther culture and both parental lines were used in the study.

Prior to germination, rice seeds were soaked into 0.7% nitric acid (HNO_3) (CAS: 7697-37-2, Lot No. 2016B3902; Junsei Chemical Co. Ltd., Tokyo, Japan) for 24 h to break the dormancy [34], followed

by incubation at 27 °C for 48 h to induce germination. Seedlings with uniform height (six seedlings per treatment per rice line) were transferred into 50 mL falcon tubes prior to $KClO_3$ application. The roots of seedlings were dipped into 5 mL of 0.1% potassium chlorate ($KClO_3$) (CAS: 3811-04-9, Lot No. BCBW5513; Sigma-Aldrich, St. Louis, MO, USA) solution (pH 5.6) and placed in a growth chamber under dark conditions for 7 days at about 25 °C [6]. The $KClO_3$ solution was replaced three days after initial supplementation by irrigation method. Control seedlings were supplemented with distilled water only. The phenotypic response was recorded 7 days after $KClO_3$ treatment and used for the construction of the linkage groups and QTL analysis. The chlorate resistance was calculated as the percentage of the [(total number of tested seedlings − number of dead seedlings in $KClO_3$)/total number of tested seedlings] × 100. Rice DH lines with a percentage of survival above or equal to 60% were scored resistant to potassium chlorate, while those with a survival percentage below or equal to 50% were scored sensitive. However, shoot inhibition was estimated as the percentage of the [(shoot length under control (SLC) − shoot length under $KClO_3$ (SL_$KClO_3$)/SLC] × 100. The latter formula was also used to estimate the root inhibition percentage [6]. Six replications were used to evaluate the phenotypic response of the DH population to potassium chlorate.

2.2. Q–Q Plot, Frequency Distribution, Correlation Analysis, and Principal Component Analysis

The Quantile–Quantile (Q–Q) plots, frequency distribution, and correlation analysis of all phenotypes of the mapping population, the pairwise kinship matrix, KASP marker density per chromosome, heat map, and principal component analysis were visualized using *qqplotr*, *heatmap.2*, *tidiverse*, *cluster*, *factoextra* (for heat map only), *GAPIT/LDheatmap*, and *ggplot2* R packages in RStudio [35,36].

2.3. Construction of Linkage Map and QTL Analysis

The genotype and phenotype data consisted of 229 KASP markers [37] and a population of 117 rice DH lines and parental lines that were used for detecting QTLs associated with chlorate resistance and constructing the genetic linkage map. Phenotypic traits studied were shoot length (SLC for control and SL_$KClO_3$ for treatment) and root length (RLC for control and RL_$KClO_3$ for treatment) under both normal (water) and $KClO_3$ treatment, shoot and root inhibition percentage, and the degree of chlorate resistance (percentage of survived seedlings). An initial formatting of phenotype and genotype raw data was done using *fread*, *pheno.raw*, *geno.raw*, and *cbind* functions in RStudio environment (v.1.2.5042, 2009–2020; RStudio Inc., Boston, MA, USA). The linkage map was constructed for a bi-parental population with IciMapping software v.4.1.0.0 using position mapping and Kosambi mapping functions [38]. The threshold of logarithm of the odds (LOD) was set as 3.0 (alpha = 0.05) to explain the probability for detecting statistically significant QTLs associated with the chlorate resistance [39].

2.4. Genomic DNA Extraction and Genotyping

The genomic DNA was extracted from leaf samples using the previously described CTAB method with slight modifications [40]. Briefly, frozen leaf samples were crushed in 1.5 mL Eppendorf tubes (e-tubes). Then, 600 µL of 2X CTAB buffer (D2026, Lot D2618U12K; Biosesang, Seongnam-si, Korea) was added and the mixture was vortexed and incubated for 30 min at 65 °C in a dry oven. A solution containing 500 µL of PCI (Phenol:Chloroform:Isoamylalcohol, 25:24:1, Batch No. 0888k0774; Sigma-Aldrich, St. Louis, MO, USA) was added, followed by gentle mixing by inversion. The tubes were centrifuged for 15 min at 13,000 rpm, and the supernatant was transferred to fresh e-tubes, followed by the addition of 500 µL of isopropanol (CAS: 67-63-0, Lot No. SHBC3600V; Sigma-Aldrich, St. Louis, MO, USA), mixing by inversion, incubation at −20 °C for 1 h, and centrifugation at 13,000 rpm for 7 min. The supernatant was removed and the pellets were washed with 70% ethanol (1 mL). Samples were centrifuged at 13,000 rpm for 2 min and ethanol was discarded, followed by drying at room temperature and re-suspension in 100 µL 1X TE buffer (Lot No. 0000278325; Promega, Madison, WI, USA).

The genotyping of parental lines for nitrate reductase (NR) and nitrate transporter (NRT) genes was done by Polymerase Chain Reaction (PCR) using OsNR-IND2194 and OsNRT-M10-22 insertion/deletion (InDel) markers. The reaction volume was 15 µL composed of 1.5 µL 10X reaction buffer, 0.8 µL 10 mM dNTP, 1 µL primers (forward and reverse), 0.1 µL Taq polymerase, and adjusted to the final volume with nuclease-free water. A 3-step cycling reaction was performed including polymerase activation at 95 °C for 5 min, strand separation at 94 °C for 20 s, annealing at 56–59 °C for 30 s for 35 cycles, extension at 72 °C for 1 min/kb, and a final extension at 72 °C for 5 min. The amplicons were separated on 3% agarose gel electrophoresis and the bands were visualized using the gel documentation system. The sequences of InDel primers can be found in Table S1.

2.5. Comparative Alignment of Coding Sequences (CDS) of Candidate Genes between Indica and Japonica Subspecies

We were also interested in investigating the similarity between homolog genes in *indica* and *japonica* subspecies in order to predict the similarity of the function of the protein they encode. Therefore, we aligned the coding sequences (CDS) of each gene, downloaded from the Nipponbare genome database (http://rice.plantbiology.msu.edu) for *japonica*, and *indica* rice genome database (http://plants.ensembl.org/Oryza_indica), using ClustalW Multiple Alignment function in BioEdit Sequence Alignment Editor [41].

3. Results

3.1. Differential Phenotypic Response of Parental Genotypes and DH Lines

The pattern of shoot growth indicated differential shoot inhibition among DH lines under potassium chlorate treatment (Figure S1A).

The parental line 93-11 (P1, *indica* subspecies) recorded an average shoot growth reduction of 45%, while the *japonica* rice cultivar Milyang352 showed about 36% shoot growth inhibition (Figure S1A). The density plot (heat map) displayed the relationship between shoot inhibition and chlorate sensitivity level (Figure S1B). The color intensity showed the degree of chlorate sensitivity of the entire DH population compared to the shoot growth inhibition pattern. Data indicated that a right (positive) skewness for shoot length and root length under control conditions was observed, which revealed the asymmetry from the normal distribution for chlorate resistant trait, while showing a negative Kurtosis relative to the mean (Table S2, Figure 1A,C,E,G). A normal distribution was observed in shoot length and root length under potassium chlorate treatment (Figure 1B,D), also supported by the Q–Q plots (Figure 1F,H). In addition, panels I to M in Figure 1 show a positive skewness for shoot and root inhibition and the chlorate resistance scores of DH lines.

Moreover, the correlation analysis revealed that there was a very weak positive correlation or non-existing correlation between shoot inhibition and chlorate resistance of DH lines (Figure 2A, Table S3). However, a strong negative correlation was observed between root inhibition and chlorate resistance (Figure 2B, Table S3).

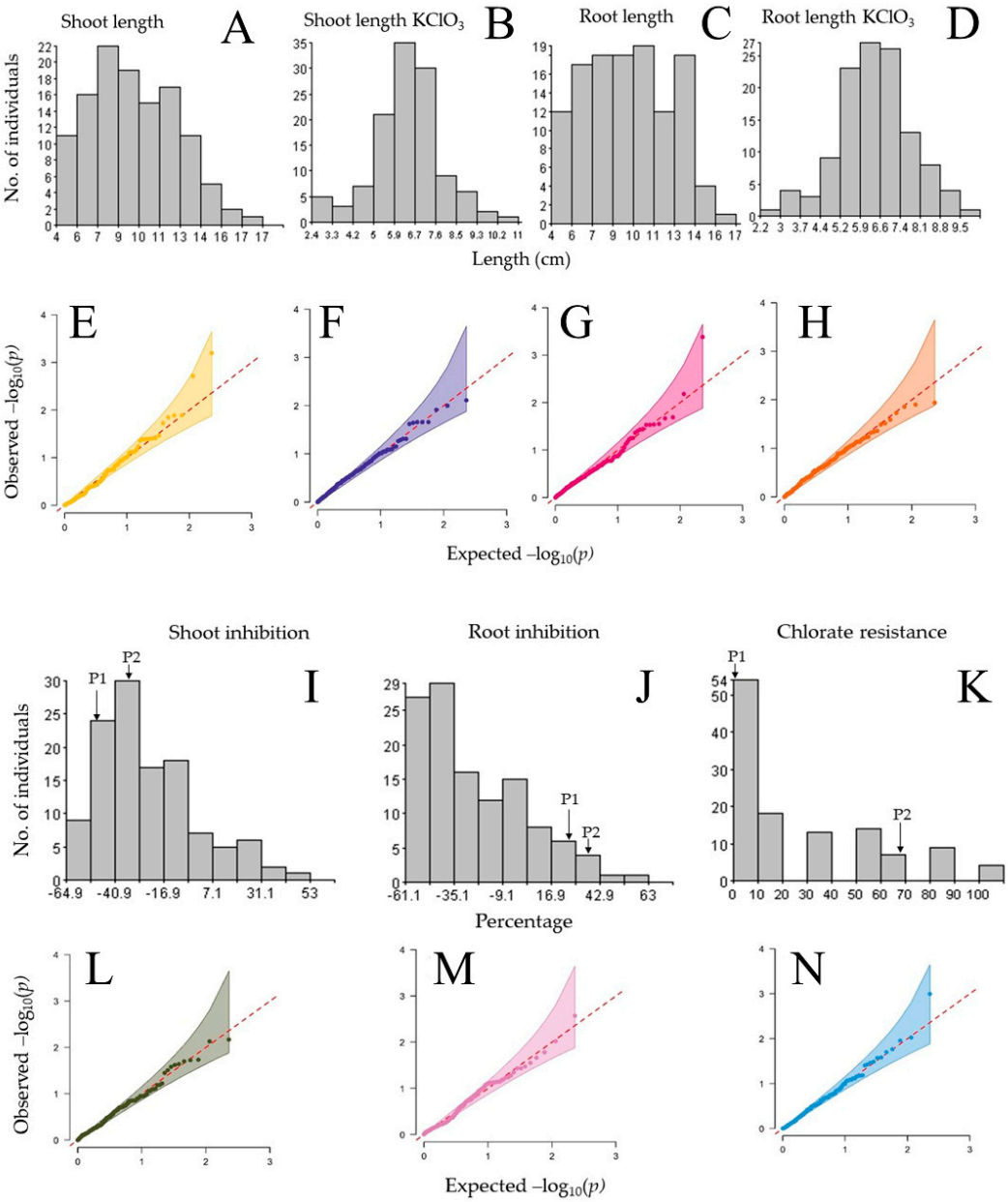

Figure 1. Trait frequency distributions under potassium chlorate treatment. (**A**) Frequency distribution of shoot length of 117 rice doubled haploid lines under normal growth conditions; (**B**) shoot length in response to 0.1% potassium chlorate ($KClO_3$) treatment; (**C**) frequency distribution of root length; (**D**) frequency distribution of chlorate resistance trait; (**E**–**H**) Quantile–Quantile (Q–Q) plots for shoot length under normal growth conditions and potassium chlorate treatment, shoot inhibition and root inhibition percentages, and chlorate resistance scores; (**I**–**K**) frequency distribution of shoot and root inhibition percentages, and chlorate resistance scores. (**L**–**N**) Q–Q plots related to shoot and root inhibition, and chlorate resistance. The red dotted lines in the Q–Q plots indicate the expected distribution under null hypothesis and the thick lines indicate the distribution from the observed associated traits. The frequency distribution and Q–Q plots were generated using *hist* function and *ggplotr* R package. P1 refers to 93-11 (parental line 1), and P2 indicates Milyang352 (parental rice line 2). −log10(p) is the log base 10 quantile-quantile (Q-Q) of the p-values (observed and expected) for traits.

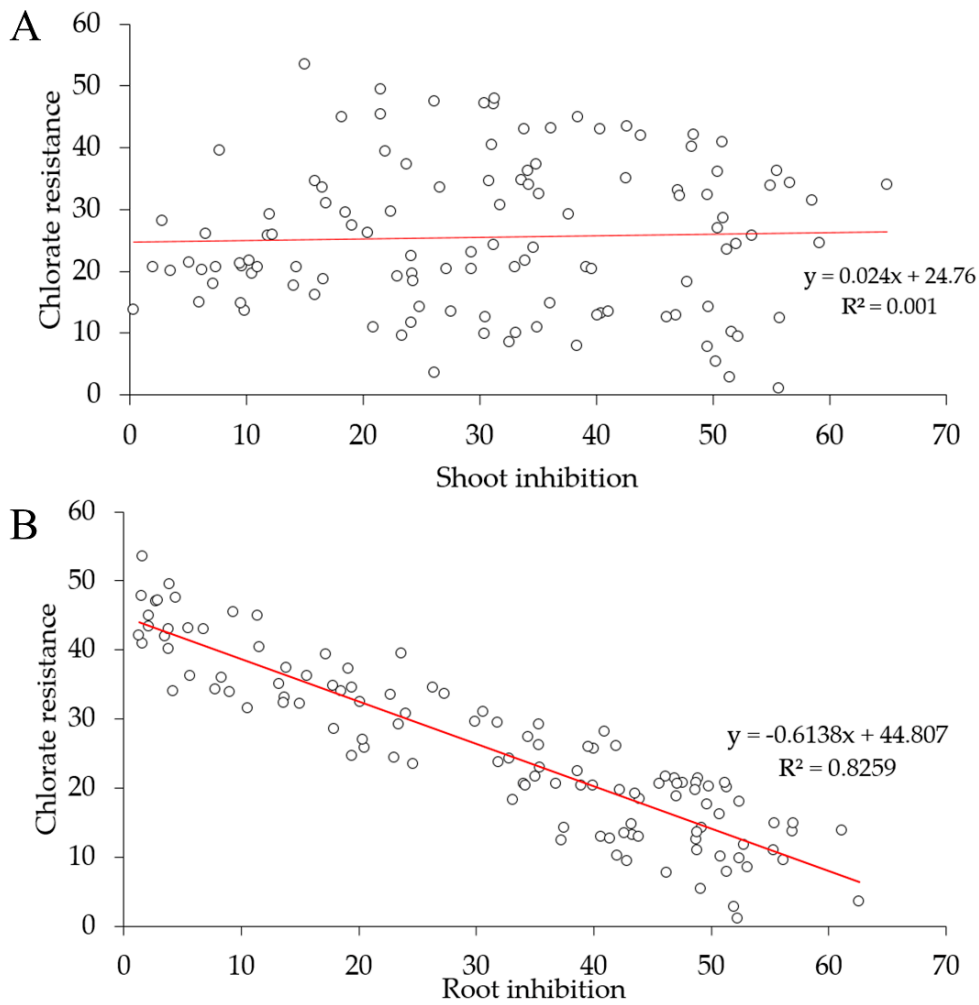

Figure 2. Correlation between seedling growth inhibition and chlorate resistance. (**A**) Predicted correlation (weak positive) between shoot inhibition percentage and chlorate resistance score and (**B**) predicted negative correlation between root inhibition and chlorate resistance phenotypes.

Roots are the first organs of plants to sense the stress when exposed to disturbed environment media conditions [42]. Therefore, we evaluated the growth pattern of roots in all DH lines and parental lines. The rice cultivar 93-11 (chlorate-sensitive) showed no significant difference in root inhibition when compared with Milyang352 (chlorate-resistant) (Figure S2A). Furthermore, a differential root growth was observed among the DH lines, indicated by the contrasting green/red patterns in Figure S2B. About 30% of the DH lines recorded an increase in root growth after being exposed to $KClO_3$ (see root inhibition (RI) column, red color strip), whereas about 60% showed an opposite pattern (RI column, green color strip). Moreover, the degree of chlorate resistance did not systematically match the shoot (SI) or root (RI) inhibition pattern. A significant difference in shoot growth inhibition between 93-11 and Milyang352 was observed (Figure 3A,B), but no significant difference was found in root growth inhibition between 93-11 and Milyang352 (Figure 3A,C). Among the 117 rice DH lines, about 24.6% scored resistant (P2 type, above 66% survival) and 75.4% scored sensitive (P1 type, below 50% survival). The parental rice cultivar 93-11 scored highly sensitive (0% survival), while Milyang352 scored resistant (66.7% survival).

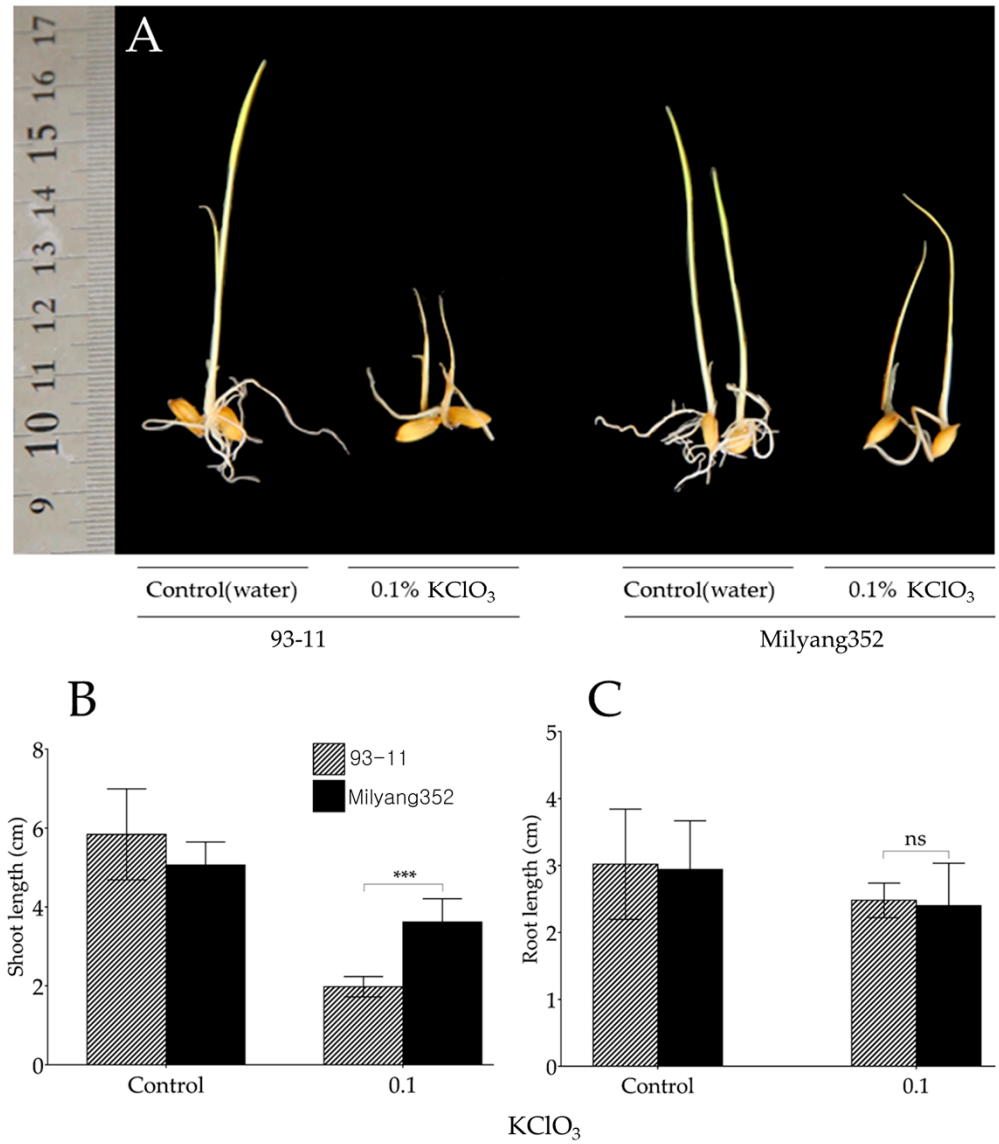

Figure 3. Distinctive phenotypic response of 93-11 (*indica*, P1) and Milyang352 (*japonica*, P2) toward potassium chlorate ($KClO_3$). (**A**) Phenotype of parental lines used for the development of doubled haploid lines under 0.1% $KClO_3$ treatment. Picture was captured 7 days after $KClO_3$ treatment by irrigation. The background of the picture was removed for clear visualization using Adobe Photoshop (Version: 13.0.1., Adobe Inc., San Jose, CA, USA) (**B**,**C**) Shoot and root growth pattern of 93-11 compared to Milyang352. *** $p < 0.001$, and ns, non-significant.

3.2. Relatedness and Principal Component Analysis (PCA)

The KASP marker-based kinship matrix (Figure 4A), also known as co-ancestry or half-relatedness, showed the distribution of coefficients of co-ancestry, with the stronger red color indicating individuals that were more related to each other or shared genomic regions derived from common parental lines (93-11 × Milyang352). The principal component analysis (PCA) revealed the contribution from three principal components (PCs) (Figure 4B). In addition, a weak correlation between shoot or root inhibition reduction and chlorate resistance level was observed (Figure 4C). Principal components 1 (PC1) and 2 (PC2) explained about 44.9% and 34.6%, respectively, of the total variation of the phenotypic response of the DH lines, which resulted in the cumulative proportion of 79.5% (Figure 4D). PC1 predicted the existence of a positive correlation between shoot inhibition and chlorate resistance, whereas PC2 suggested a negative correlation between root inhibition and chlorate resistance.

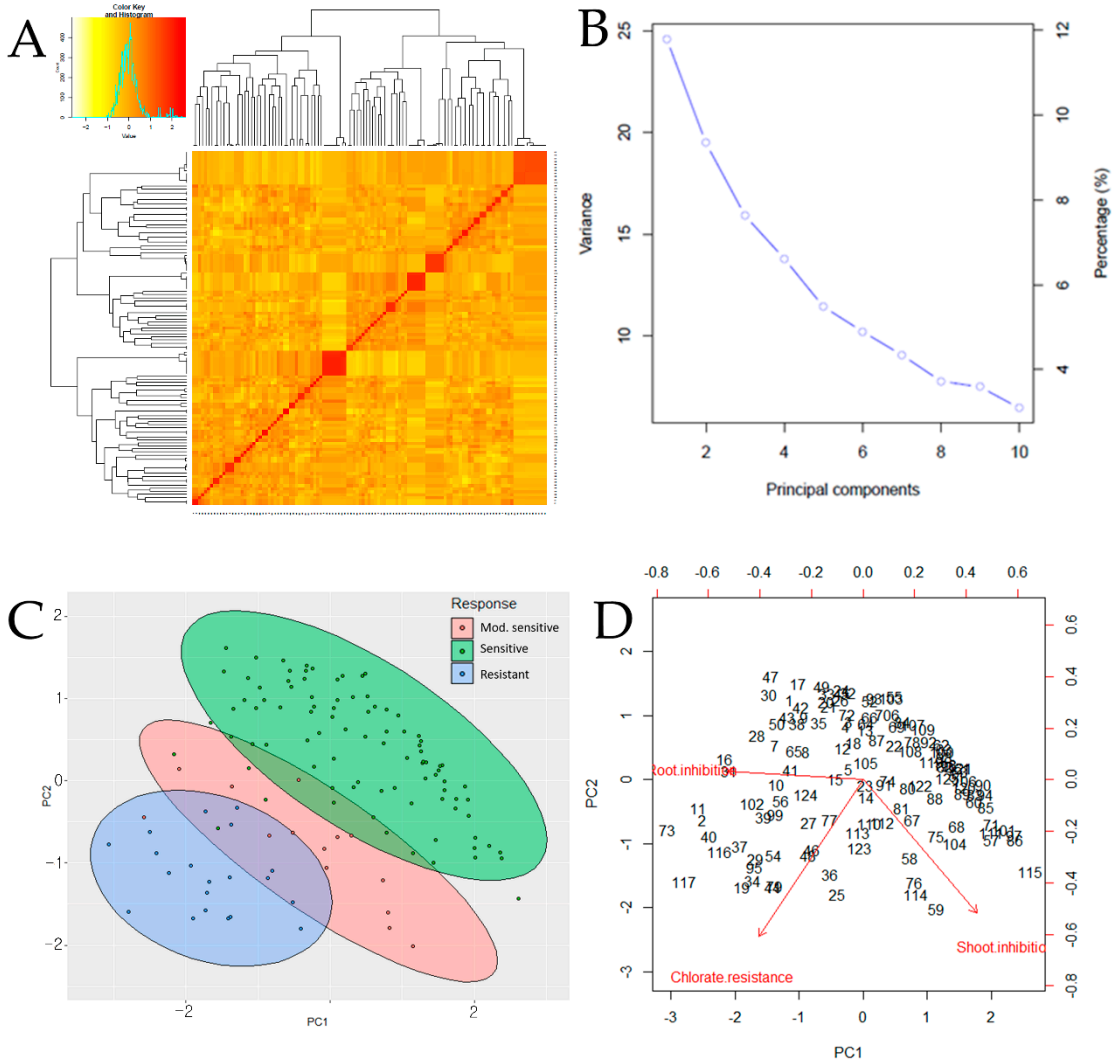

Figure 4. Kinship matrix and principal component analysis (PCA) results. (**A**) The heat map of pairwise kinship matrix values based on 229 Kompetitive Allele-Specific PCR (KASP) markers on 117 rice doubled haploid (DH) lines according to VanRaden algorithm. The color histogram indicates the distribution of coefficients of co-ancestry, with the stronger red color showing the DH lines more related to each other, (**B**) eigenvalue accumulation variance among principal components (PCs) revealed contribution to only 3 PCs, (**C**) graphic scattered plot of PCA of 117 DH lines, calculated from 229 KASP markers and clustered. Resistant, moderately sensitive, and sensitive lines are respectively grouped in blue (bottom ellipse), brown (middle ellipse), and green (top ellipse), and (**D**) principal components with correlation between variables.

3.3. Novel QTL for Chlorate Resistance Detected on Chromosome 3 in Rice

The result of linkage analysis and QTL mapping revealed the detection of a novel putative QTL associated with chlorate resistance on chromosome 3 at 136 cM (Table 1, Figure 5). KASP markers flanking the QTL *qCHR-3* were ah03001094 and id3005168. The distribution of KASP markers over 11 linkage groups (LG) was even, with LG3 and LG7 being the most densely covered (Figure S3).

Table 1. Quantitative Trait Locus (QTL) associated with chlorate resistance in rice.

Trait	QTL (a)	Chr (b)	Position (cM) (c)	Left Marker (d)	Right Marker (e)	LOD (f)	PVE (%) (g)	Add (h)
Chlorate resistance	*qCHR-3*	3	136	ah03001094	id3005168	4.1	14.92	−11.975

(a) Detected QTL name, (b) chromosome number, (c) absolute position of the QTL from top of the linkage map in centimorgan (cM), (d) left flanking KASP marker, (e) right flanking KASP marker of the QTL position, (f) logarithm of odds scores above the threshold of 3.0, (g) phenotypic variation explained (PVE) by the QTL, and (h) additive effect: the negative value indicates that the allele from Milyang352 increases the trait value.

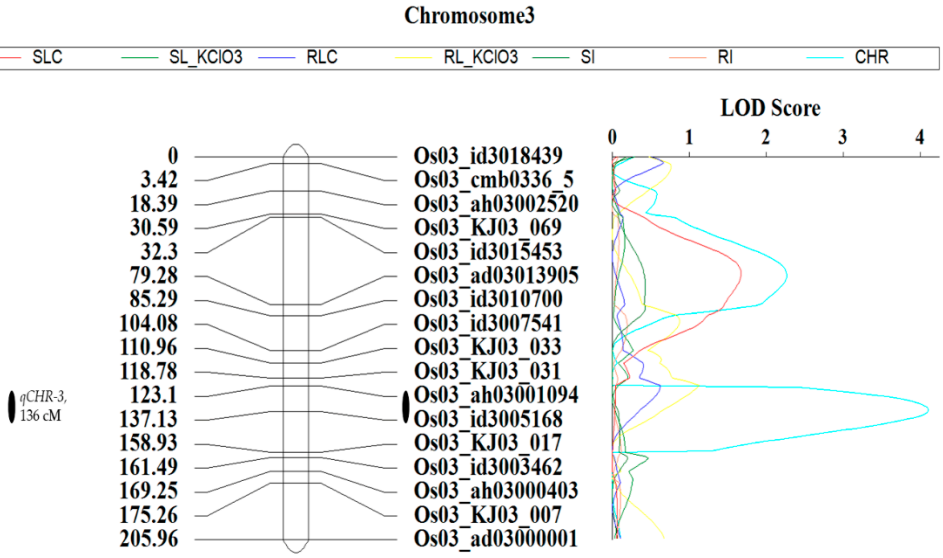

Figure 5. Linkage map and identified QTL associated with chlorate resistance in rice. The significant QTL for chlorate resistance was detected on chromosome 3 (136 cM) in rice. KASP markers associated with the QTL *qCHR-3* with logarithm of the odds (LOD) above the threshold set at 3 were Os03_ah03001094 (left, 126.5 cM) and Os03_id3005168 (right, 146.5). Left side of the linkage map shows the position of the mapped KASP markers in cM (right side). The LOD profile is displayed on the right side of the linkage map. SLC, shoot length with no treatment; SL_KClO3, shoot length with treatment; RLC, root length without treatment; RL_KClO3, root length after treatment; SI, shoot inhibition percentage; RI, root inhibition percentage; and CHR, chlorate resistance in percentage.

Candidate genes (Table 2) were pooled from the region covering about 300 kb, the closest to the right marker (the closest to the QTL) flanking the right side of the detected QTL.

Large deletion mutations between *indica* and *japonica* were discerned in eight genes (Table 2).

3.4. InDel Markers Amplified Polymorphic Bands between Parental Lines

After their exposure to potassium chlorate (0.1%), parental rice lines 93-11 (P1) and Milyang352 (P2) showed distinctive phenotypic responses toward $KClO_3$, sensitive and tolerant, respectively. We were further interested to investigate the presence of resistance alleles of previously reported genes controlling chlorate resistance in plants, such as nitrate reductase (NR) and nitrate transporter (NRT) [8,10], using the InDel markers, OsNR-IND2194 and OsNRT-M10-22. The genotyping results of parental lines indicated that both the InDel markers amplified polymorphic bands between the parental lines. OsNR-IND2194 amplified a band size of 200 bp in 93-11 (P1) and 188 bp in Milyang352 (P2) (Figure 6A). OsNRT-M10-22 amplified polymorphic bands of 165 bp in 93-11 and 213 bp in Milyang352 (Figure 6C,D). The amplified band sizes matched the deletion of nucleotides observed through in silico analysis of NR- and NRT-coding sequences (CDS) in *indica* and *japonica* databases (Figure 6B,D).

Table 2. List of candidate genes.

MSU ID (Nipponbare)	*Indica* Database (Ensembl)	Description	Remarks	Similar Papers
LOC_Os03g18170	BGIOSGA012400	Protein kinase domain-containing protein, expressed	162 bp deletion in *indica*	[43]
LOC_Os03g18380	BGIOSGA010930	Ubiquitin-activating enzyme E1	357 bp deletion in *japonica*	[44,45]
LOC_Os03g18500	BGIOSGA010922	Mitochondrial import inner membrane translocase subunit tim22	100% similar	[46]
LOC_Os03g18550	BGIOSGA012419	Mitochondrial carrier protein domain-containing protein	100% similar	[46]
LOC_Os03g18630	BGIOSGA012425	Leucine-rich repeat family protein, receptor-like kinase RHG1, putative, expressed	100% similar	[47]
LOC_Os03g18690	BGIOSGA012432	26S protease regulatory subunit 4 homolog (26S proteasome subunit AtRPT2a)	72 bp deletion in *indica* and 102 bp deletion in *japonica*	[45]
LOC_Os03g18700	BGIOSGA010916	Sel1-like domain containing protein (Sel1-like repeats); these represent a subfamily of TPR (tetratricopeptide repeat) sequences.	306 bp deletion in *indica*, and 30 bp deletion in *japonica*	[48]
LOC_Os03g18790	BGIOSGA010911	Senescence-associated gene 20 (SAG20) putative, expressed. Cys-rich family protein (DUF2985) and PLAC8 family domains. This family includes the placenta-specific gene 8 protein.	100% similar	[49]
LOC_Os03g19290	BGIOSGA012456	Mitochondrial import inner membrane translocase, subunit Tim17/22 family protein, putative, expressed	27 bp deletion in *japonica*	[46]
LOC_Os03g19380	BGIOSGA012463	Calvin cycle protein CP12, putative, expressed. A chloroplast protein that regulates the Calvin cycle responsible for CO_2 assimilation.	100% similar	[50,51]
LOC_Os03g19420	BGIOSGA010884	Nicotianamine synthase 2, putative, expressed	100% similar	[52,53]
LOC_Os03g19500	BGIOSGA012473	Ubiquitin-conjugating enzyme/RWD-like domain containing protein	3 bp deletion in *indica*	[45]
LOC_Os03g20980	BGIOSGA010820	Zinc finger, RING-type domain-containing protein. Zinc finger, C3HC4 type domain-containing protein, expressed.	51 bp deletion in *indica* and 3 bp deletion in *japonica*	[54]
LOC_Os03g21060	BGIOSGA026407	NAC domain-containing protein 29 (ANAC029) (NAC2) (NAC-LIKE, ACTIVATED BY AP3/PI protein) (NAP). No apical meristem protein, putative, expressed.	156 bp deletion in *indica* and 12 bp deletion in *japonica*	[55–57]
LOC_Os03g21090	BGIOSGA010814	Axi1 auxin-independent growth promoter protein, putative, expressed	100% similar	[58,59]
LOC_Os03g21490	BGIOSGA010800	ABC transporter, ATP-binding component. ABC transporter I family member 6, chloroplastic	100% similar	[60,61]

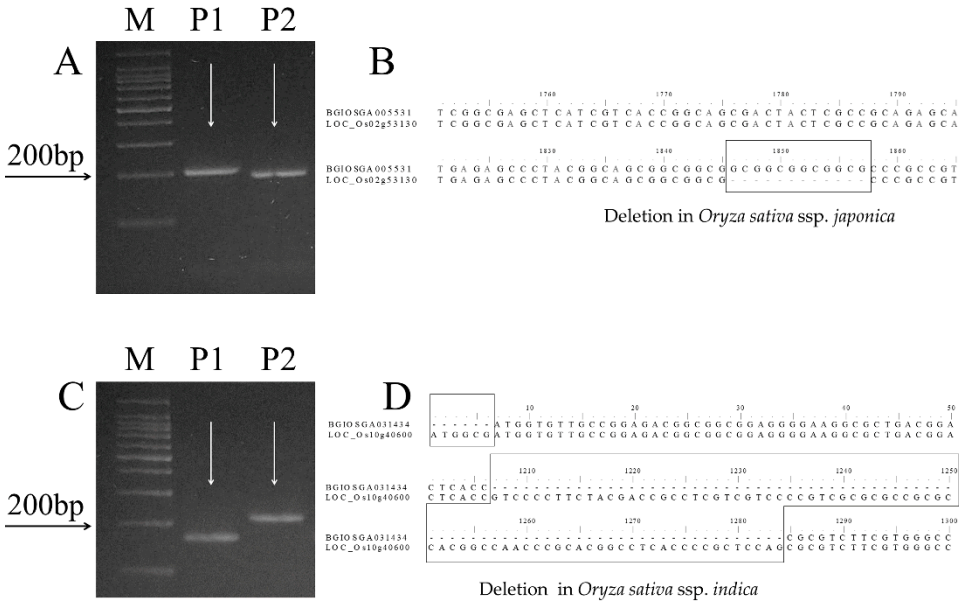

Figure 6. Genotyping of rice cultivars 93-11 (P1, *indica*) and Milyang352 (P2, *japonica*) using nitrate reductase (NR) and nitrate transporter (NRT) insertion/deletion (InDel) markers. (**A**) OsNR-IND2194 amplified a band size of 200 bp in 93-11 (*indica*) and 188 bp in Milyang352 (*japonica)*, (**B**) NR-coding sequence alignment showing deletion of 12 nucleotides (coding for alanine, 4 residues) in *Oryza sativa* ssp. *japonica*, (**C**) OsNR-M10-22 amplified a band of 165 bp in 93-11 and 213 bp in Milyang352, and (**D**) NRT-coding sequence alignment showing big deletion in *Oryza sativa* ssp. *indica*. M: ladder, 100 bp. Bands were separated using 3% agarose gel electrophoresis. Coding sequences of genes were downloaded from Nipponbare genome database (http://rice.plantbiology.msu.edu) for *japonica*, and *indica* rice genome database (http://plants.ensembl.org/Oryza_indica), using ClustalW Multiple Alignment in BioEdit Sequence Alignment Editor.

4. Discussion

Genotypes exhibiting a high degree of chlorate resistance are expected to have low nitrogen use efficiency (NUE), while those showing a sensitive phenotypic response toward potassium chlorate ($KClO_3$) are expected to have high NUE [8]. The rice cultivar 93-11 harbors the *indica* allele of nitrate reductase (*OsNR2*), which was reported to promote NO_3 uptake, while interacting with the nitrate transporter, *OsNRT1.1B*, and improving the NUE [10]. Here, we reported that the *indica* cultivar 93-11 (P1) was highly sensitive to $KClO_3$ treatment, whereas *japonica* cultivar Milyang352 (P2) showed a resistant phenotype. We observed that about 75.6% of the mapping population scored sensitive and about 24.4% showed a resistant phenotype, as explained by principal components PC1 and PC2 (Figure 4C). Moreover, shoot inhibition showed a very weak positive correlation that could also be regarded as a non-existing correlation with the recorded chlorate resistance of DH lines (Figure 4D), which implied that the degree of chlorate resistance of the studied DH lines was independent of shoot inhibition in response to $KClO_3$. Under the same conditions, a strong negative correlation was observed between root inhibition and chlorate resistance. Therefore, it may be suggested that a high degree of chlorate resistance could be explained in part by the low reduction in root inhibition under $KClO_3$ treatment.

In addition, the phenotypic records of all traits and the genotype data of the 117 DH lines and parental lines were used to perform a QTL analysis. Of the seven traits evaluated, one significant QTL for chlorate resistance was detected on chromosome 3 in rice. The QTL, *qCHR-3* located at 136 cM, is flanked by KASP markers ah03001094 (left, 126.5 cM) and id3005168 (right, 146.5 cM), the latter being closer to the QTL. The resistance allele of the detected QTL was contributed by Milyang352. This QTL counted for 14.9% of the observed phenotypic variation. In addition, the genetic region covered

by *qCHR-3* harbors genes identified as interesting candidate genes. From their specific functional annotations and conserved domains, these candidate genes resemble in their putative functions, previously reported genes from the same family or belonging to other protein families, for their role in the regulation of nitrogen metabolism or photosynthetic process in plants (Table 2). Previous studies identified genetic loci associated with chlorate resistance and nitrogen use efficiency (NUE) in rice at chromosome 2 (*qCR-2*) [9]. Moreover, fine mapping of these loci allowed the identification of genes such as nitrate reductase (*OsNR2*, Chr2) and nitrate transporter (*OsNRT1.1B*, Chr10) [62,63]. Furthermore, Teng and his colleagues [6] reported earlier the detection of QTLs associated with chlorate resistance (*qCHR-2*, *qCHR-8*, and *qCHR-10*) in an F1 DH population. These genes were reported for playing an important role in the initial steps of nitrate assimilation and transport in plants, one acting to convert nitrate (NO_3) to nitrite (NO_2) and the other transporting NO_3 throughout the cell. The QTLs, *qCHR-2*, *qCHR-8*, and *qCHR-10*, were reported to explain about 26.5%, 12.6%, and 12.6% of the phenotype variance [6]. The results of genotyping (Figure 6) indicated that the parental lines 93-11 (*indica*) and Milyang352 (*japonica*) amplified polymorphic bands of both the OsNR-IND2194 and OsNRTM-10-22 markers, matching the deletion patterns observed between *indica* and *japonica* through in silico analysis.

From another perspective, NUE is regarded as a complex and integrated mechanism, which can be controlled at different levels, under the influence of various cellular components and metabolic processes [64–68]. It is said that carbon and nitrogen metabolism are intimately related, considering the fact that about 55% of the net carbon in plants, is allocated to nitrogen assimilation and metabolism [69]. Moreover, the authors supported that the energy and carbon supplied from the photosynthetic electron transport chain (ETC), carbon dioxide (CO_2) fixation, respiration, or tricarboxylic acid (TCA) cycle are shared by carbon and nitrogen metabolisms [67,70].

Thus, among the candidate genes listed in Table 2, we identified genes coding for ubiquitin-activating enzyme E1 [44,45], ubiquitin-conjugating enzyme, 26S proteasome AtRPT2a [45], senescence-associated gene 20 (*SAG20*) [49], Calvin cycle protein CP12 [50,51], etc., which share specific conserved domains with previously reported genes for being linked to the regulation of the nitrogen metabolism in plants. Therefore, this QTL could serve as a target locus for breeding for NUE and functional studies. In addition, the large deletion mutations found in the coding sequences of some of the selected candidate genes could be exploited to investigate their differential transcriptional regulation between *indica* and *japonica* subspecies.

5. Conclusions

Understanding chlorate resistance and the genes involved in the process would help to better comprehend the mechanism underlying nitrogen metabolism in plants and develop rice varieties with increased efficiency in the use of nitrogen. The present study identified a novel QTL associated with chlorate resistance in rice using Kompetitive Allele-Specific PCR (KASP) markers. This QTL harbors candidate genes that could play important roles in the regulation of nitrogen metabolism in rice with regard to their predicted functions. A downstream breeding program and functional analysis would help to identify key genes and elucidate their function.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0472/10/8/360/s1, Figure S1: Shoot growth pattern relative to chlorate resistance, Figure S2: Root growth pattern relative to the level of chlorate resistance, Figure S3: Density of KASP markers across the rice genome, Table S1: Sequences of InDel makers, Table S2: Statistical output of analyzed traits in DH population, Table S3: Correlation analysis between traits.

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