Resistance to Multiple Temperate and Tropical Stem and Sheath Diseases of Rice

Juan E. Rosas, Sebastián Martínez, Pedro Blanco, Fernando Pérez de Vida, Victoria Bonnecarrère, Gloria Mosquera, Maribel Cruz, Silvia Garaycochea, Eliana Monteverde, Susan McCouch, Silvia Germán, Jean-Luc Jannink, and Lucía Gutiérrez*

ABSTRACT

Stem rot and aggregated sheath spot are the two major stem and sheath diseases affecting rice (Oryza sativa L.) in temperate areas. A third fungal disease, sheath blight, is a major disease in tropical areas. Resistance to these diseases is a key objective in rice breeding programs but phenotyping is challenged by the confounding effects of phenological and morphological traits such as flowering time (FT) and plant height (PH). This study sought to identify quantitative trait loci (QTL) for resistance to these three diseases after removing the confounding effects of FT and PH. Two populations of advanced breeding germplasm, one with 316 tropical japonica and the other with 325 indica genotypes, were evaluated in field and greenhouse trials for resistance to the diseases. Phenotypic means for field and greenhouse disease resistance, adjusted by FT and PH, were analyzed for associations with 29,000 single nucleotide polymorphisms (SNPs) in tropical japonica and 50,000 SNPs in indica. A total of 29 QTL were found for resistance that were not associated with FT or PH. Multilocus models with selected resistance-associated SNPs were fitted for each disease to estimate their effects on the other diseases. A QTL on chromosome 9 accounted for more than 15% of the phenotypic variance for the three diseases. When resistance-associated SNPs at this locus from both the tropical japonica and indica populations were incorporated into the model, resistance was improved for all three diseases with little impact on FT and PH.

Core Ideas

- Reaction to sheath blight, stem rot, and aggregated sheath spot were tested in 641 tropical japonica and indica rice lines.
- Disease resistance was mapped independently from flowering time and plant height.
- Quantitative trait loci of major effect for resistance to the three diseases were found.
- A multiple disease resistance quantitative trait locus was found on chromosome 9 across tropical japonica and indica populations.


*Corresponding author (gutierrezcha@wisc.edu).

Abbreviations: FT, flowering time; GWAS, genome wide association study; INIA, Instituto Nacional de Investigación Agropecuaria; LD, linkage disequilibrium; PH, plant height; PVE, phenotypic variance explained; QTL, quantitative trait locus; SNP, single nucleotide polymorphism.
There is a large gap between yield potential and the yields obtained by farmers in most rice-growing countries (Laborte et al., 2012). Rice diseases explain a substantial proportion of this yield gap (Savary et al., 2000; Van Nguyen and Ferrero, 2006). In temperate and subtropical areas like the Southern Cone of South America, the yield gap has been reduced significantly over the last decades thanks to the widespread adoption of modern cultivars and technology (Martínez et al., 2014; Pittelkow et al., 2016). However, high yields in these areas rely on the intensive use of fungicides, as most of the top cultivars and technology (Martínez et al., 2014; Pittelkow et al., 2016). The objective of the present study is to map the genetic resistance to the three fungal pathogens, *T. cucumeris*, *N. oryzae*, and *R. oryzae-sativae*, and the identification of resistance QTL for all three diseases in diverse genetic backgrounds is needed to facilitate selection in rice breeding and accelerate genetic gain for these traits.

Genome-wide association studies (GWAS) are designed to map QTL in populations with diverse genetic backgrounds and levels of relatedness (Jannink et al., 2001). Genome-wide association studies have been used to map QTL in a variety of crops with diverse germplasm collections (Yu et al., 2008; Zhao et al., 2011; Zhu et al., 2008) or breeding populations (Begum et al., 2015; Liang et al., 2016). The approach has enabled the identification of QTL for resistance to multiple diseases (Gurung et al., 2008) or breeding populations (Begum et al., 2015; Liang et al., 2016). Multilocus models may be used to jointly test the effect of all QTL discovered by GWAS (Locatelli et al., 2013; Gutierrez et al., 2015). The objective of the present study is to map the genetic resistance to the three fungal pathogens, *T. cucumeris*, *N. oryzae*, and *R. oryzae-sativae*, independent of FT and PH, in tropical *japonica* and *indica* rice breeding germplasm. This is the first study to focus on the genetics of resistance to *R. oryzae-sativae* and the first report on one QTL for sheath blight resistance colocalizing with the semidwarf gene *sd-1* in chromosome 1. In most cases, it is impossible to distinguish between linkage and pleiotropy at those QTL (Wiesner-Hanks and Nelson, 2016). Proposed strategies for minimizing the confounding effect of FT and PH for evaluating resistance to confounded diseases include the use of controlled phenotyping methodologies such as microchambers and mist chambers (Jia et al., 2007; Liu et al., 2013), rating systems that are not influenced by PH (Zeng et al., 2015), mapping populations that do not segregate for FT or PH (Liu et al., 2013), and using FT and PH as covariates for correcting phenotypic disease scores (Paillard et al., 2004) or using FT and PH as covariates in the QTL mapping analysis (Nelson et al., 2012).

Resistance to *T. cucumeris*, *N. oryzae*, and presumably to *R. oryzae-sativae*, are quantitative traits governed by multiple loci of minor effect (Lanoiselet et al., 2007; Ni et al., 2001; Srinivasachary et al., 2013). Several QTL for resistance to *T. cucumeris* have been identified in a variety of crosses and populations, and across all rice chromosomes, but predominantly in *indica* genetic backgrounds (Srinivasachary and Savary, 2011). The confounding effect of PH and FT on disease resistance has led to the identification of many false positive QTL for these traits (Zeng et al., 2015). The need for studies on the genetics of nonescape and physiological resistance to sheath blight have been stressed (Lore et al., 2013; Zeng et al., 2015), but the genetics of resistance to stem rot and aggregated sheath spot have scarcely been studied. To our knowledge, there is only one report on QTL for resistance to stem rot (Ni et al., 2001) and no reports for resistance to aggregated sheath spot. Thus a better understanding of the genetics of resistance to *N. oryzae* and *R. oryzae-sativae*, and the identification of resistance QTL for all three diseases in diverse genetic backgrounds is needed to facilitate selection in rice breeding and accelerate genetic gain for these traits.

T. *cucumeris*, *N. oryzae*, and *R. oryzae-sativae* are sclerotial pathogens with similar life cycles and display predominantly monocyclic infections, sporulating in rice plants only late in the season under field conditions (Lanoiselet et al., 2005; Ou, 1985). Sclerotia and mycelium can overwinter in rice stubble and soil, and infect rice sheaths at the water line (Krause and Webster, 1973; Lanoiselet et al., 2007; McKenzie et al., 1994). Yield losses have been quantified as up to 22% as a result of *N. oryzae* (Krause and Webster, 1973), and 20% for *R. oryzae-sativae* (Lanoiselet et al., 2005). Hence, susceptibility to stem and sheath diseases is a major concern for breeding programs for large and small yield gap conditions, and for tropical and temperate rice growing regions.

Genome-wide association studies (GWAS) are designed to map QTL in populations with diverse genetic backgrounds and levels of relatedness (Jannink et al., 2001). Genome-wide association studies have been used to map QTL in a variety of crops with diverse germplasm collections (Yu et al., 2008; Zhao et al., 2011; Zhu et al., 2008) or breeding populations (Begum et al., 2015; Liang et al., 2016). The approach has enabled the identification of QTL for resistance to multiple diseases (Gurung et al., 2008) or breeding populations (Begum et al., 2015; Liang et al., 2016). Multilocus models may be used to jointly test the effect of all QTL discovered by GWAS (Locatelli et al., 2013; Gutierrez et al., 2015). The objective of the present study is to map the genetic resistance to the three fungal pathogens, *T. cucumeris*, *N. oryzae*, and *R. oryzae-sativae*, independent of FT and PH, in tropical *japonica* and *indica* rice breeding germplasm. This is the first study to focus on the genetics of resistance to *R. oryzae-sativae* and the first report on
GWAS for the identification of QTL for resistance to *N. oryzae* and *R. oryzae-sativae*. The QTL identified in this work will contribute to improving resistance to the three studied diseases in both tropical *japonica* and *indica* genetic backgrounds.

**MATERIALS AND METHODS**

**Plant Material and Phenotyping**

Resistance to the three fungal pathogens, *T. cucumeris*, *N. oryzae*, and *R. oryzae-sativae*, was individually evaluated in 641 advanced inbred lines (325 tropical *japonica* and 316 *indica*) from the National Agricultural Research Institute (INIA) of Uruguay rice breeding program. Resistance to *N. oryzae* and *R. oryzae-sativae* was evaluated in both greenhouse and field trials in Uruguay, whereas resistance to *T. cucumeris* was evaluated in greenhouse trials in Colombia. Greenhouse experiments for *N. oryzae* and *R. oryzae-sativae* have been described elsewhere (Rosas et al., 2016). Briefly, two trials for *N. oryzae* and three trials for *R. oryzae-sativae* were conducted from 2012 to 2014 at INIA Treinta y Tres Experimental Station, Treinta y Tres, Uruguay (33°15′S, 54°25′W). The cultivars ‘El Paso 144’, ‘INIA Olimar’, ‘INIA Tacuari’, ‘Parao’, and ‘Lemont’ were used as replicated checks. Resistance to *T. cucumeris* was evaluated in 2015 in two greenhouse trials at CIAT, Valle del Cauca, Colombia (3°29′N, 76°21′W) following the mist-chamber method (Jia et al., 2007). The varieties ‘Oryzica 3’ and Lemont were used as replicated resistant and susceptible checks, respectively. For all greenhouse experiments, Federer’s unreplicated design (Federer and Raghavarao, 1975) with augmented checks in randomized complete blocks was used, and the environmental conditions were 28-18°C day/night, 80-90% relative humidity and 12 h light time. Ten seeds were sown in 12-cm diameter pots.

After plant emergence, four (for *N. oryzae* and *R. oryzae-sativae*) or two (for *T. cucumeris*) plants per pot were grown for inoculation. At the three-leaf stage plants were inoculated with 5-mm agar discs with fresh mycelium at the base of the stem. An additional disc in the third leaf was used for *T. cucumeris*. Diseases were scored at 45 d after inoculation using the percentage of relative lesion length based on modifications of IRRI’s standard evaluation system for rice (IRRI, 2002) described in Martínez (2016) and depicted in Supplemental Fig. S1 and Supplemental Fig. S2 for *N. oryzae* and *R. oryzae-sativae*, respectively, and in Jia et al. (2007) for *T. cucumeris*. Stem width at inoculation time and phenological stage at scoring time were recorded. Phenological stage was used as a proxy for FT in the greenhouse experiments.

Field trials for *N. oryzae* and *R. oryzae-sativae* were performed from 2009 to 2013 at the Paso de la Laguna Experimental Unit, Treinta y Tres, Uruguay (33°54′S, 54°38′W). Trials from 2009 to 2012 used naturally infected six-row plots (1.2 by 3.5 m) laid out in a randomized complete block design with three replicates. Three points in each plot were visually assessed for *N. oryzae* and *R. oryzae-sativae* symptoms separately, rating disease severity on the basis of a 0- to 9 scale (IRRI, 2002) depicted in Supplemental Fig. S1. The 2013 field trials were artificially inoculated hill plots of ~10 adult plants in an augmented α-lattice design (Piepho et al., 2006) with six replicates. Plots were treated with 0.6 g m⁻² (*N. oryzae* trials) or 1.8 g m⁻² (*R. oryzae-sativae* trials) of inoculum sprinkled from about 50 cm over the floodwater surface at the beginning of the tillering stage. Flowering time and plant height at maturity were measured in all field experiments and used as covariates to remove confounding effects on disease resistance.

Adjusted phenotypic means were estimated using the model in Eq. [1]:

\[
Y_{ijmn} = \mu + \gamma_i + G_j + \beta_1 X_1 + \beta_2 X_2 + R_{m(i)} + C_{n(i)} + \varepsilon_{ijmn}, [1]
\]

where \(Y_{ijmn}\) is the response variable; \(\mu\) is the intercept; \(\gamma_i\) is the random block effect with \(\gamma_i \sim N(0, \sigma^2_{\gamma})\); \(G_j\) is the genotypic effect; \(X_1\) and \(X_2\) are the covariates in the \(i^{th}\) genotype and the \(j^{th}\) block, and \(\beta_1\) and \(\beta_2\) are the regression slopes of the covariates (FT and stem width for greenhouse trials; FT and PH for field trials, and none for estimation of the phenotypic means of FT and PH); \(R_{m(i)}\) and \(C_{n(i)}\) are the random row and column effects nested within blocks, with \(R_{m(i)} \sim N(0, \sigma^2_{R})\) and \(C_{n(i)} \sim N(0, \sigma^2_{C})\); and \(\varepsilon_{ijmn}\) is the residual. The genotypic effect was modeled as \(G_j = g_k + c_p\), where \(g_k\) is the effect of the \(k^{th}\) genotype, and \(c_p\) is the fixed effect of the \(p^{th}\) check. For estimation of genetic variances, \(g_k\) was modeled as a random effect with \(g_k \sim N(0, \sigma^2_{G})\). For Pearson correlation analysis, \(g_k\) was modeled as a fixed effect. This notation for genotypic effects follows that of Eckermann et al. (2001) and Verbyla et al. (2003). To illustrate the consequences of not correcting for FT and PH, a naïve model without covariates was also used to estimate the disease-adjusted means from field experiments. Generalized heritability was estimated as \(h^2_{BLUP} = \frac{\hat{v}_{BLUP}}{\hat{v}_{BLUP} + \hat{\sigma}^2}\), where \(\hat{v}_{BLUP}\) is the mean pairwise variance error of the genotypic best linear unbiased prediction (Cullis et al., 2006), and the SE was estimated with a bootstrap data resampling technique, as recommended by Holland et al. (2003). Phenotypic means were averaged and weighted by the generalized heritability of each individual trial. Two-sample Student’s \(t\)-tests were performed to compare the weighted phenotypic means of each trait in tropical *japonica* vs. *indica* populations. Analyses were performed in R with the packages lme4 (Bates et al., 2015), lsmeans (Lenth, 2016), arm (Gelman et al., 2016), and boot (Canty and Ripley, 2015).

**Fungal Isolates and Inoculum Production**

Monosclerotial strains were isolated from naturally infected rice plants and soil, and selected to have intermediate aggressiveness on rice. Strain SO-Samba (*N. oryzae*) was isolated in 2001 from the temperate *japonica* rice cultivar Samba in naturally infected experimental plots at Paso de la Laguna Experimental Unit. Strain
ROS-17 (R. oryzae-sativae) was obtained from soil after cultivation with the tropical japonica rice cultivar INIA Tacuari in Paso de la Laguna Experimental Unit in 2003. The SO-Samba and ROS-17 strains caused good discrimination among rice genotypes, and are maintained at the Culture Collection of the Plant Pathology Laboratory of INIA Treinta y Tres (Rosas et al., 2016). Strain 1953–1 (T. cucumeris) was collected in 1985 in Tolima, Colombia, and is maintained at the Culture Collection of the CIAT Pathology Laboratory. For greenhouse experiments, isolates were grown for 5–7 d at 25°C in 90-mm Petri dishes containing potato dextrose agar (Oxoid, Basingstoke, United Kingdom) for N. oryzae and R. oryzae-sativae, or rice bran agar for T. cucumeris. Sclerotia for inoculation of field trials in 2013 were produced separately from sterilized mixtures of 2:1 rice seeds and rice hulls that were inoculated with 7-d-old mycelia, incubated for 25 d at 23°C, dried at 40°C, and conserved at 4°C until use (Krause and Webster, 1973).

Genotyping

Rice DNA was isolated in the Biotechnology Unit of INIA (Canelones, Uruguay) with the DNeasy kit (Qiagen, Hilden, Germany) and genotyped using genotyping-by-sequencing with the ApeK1 enzyme in the Biotechnology Resource Center at Cornell University (Ithaca, NY). Single nucleotide polymorphism NP calling was performed with the TASSEL version 3.0 genotyping-by-sequencing pipeline (Bradbury et al., 2007). Single nucleotid polymorphisms were aligned to the Nipponbare reference genome, Rice Genome Annotation Project release 7 (Kawahara et al., 2013), with Bowtie 2 (Langmead and Salzberg, 2012). Missing SNP data were imputed with the FILLIN algorithm (Swarts et al., 2014) and SNPs with a minor allele frequency <1%. >50% of missing data or both were removed. The final genotypic matrices contained 49,589 SNPs for indica and 28,850 SNPs for tropical japonica.

Population Structure, GWAS Scanning, Multilocus and Linkage Disequilibrium Block Analyses

Because of the deep population structure existent within the O. sativa species, indica and tropical japonica populations were analyzed separately. To assess population structure within each subspecies, principal component analysis was performed on each genotypic matrix. GWAS and multilocus analyses were tested by fitting the mixed linear model $y = X\beta + Zu + e$, where $y$ is a vector of adjusted phenotypic means; $\beta$ is a vector of fixed effects (single SNPs for the GWAS scan and multiple selected SNPs for the multilocus analysis); $u$ is a vector of random genotypic effects with $u \sim N(0, K\sigma^2_u)$; $e$ is a vector of residual effects with $e \sim N(0, I\sigma^2_e)$; $X$ and $Z$ are incidence matrices that relate $y$ to $\beta$ and to $u$, respectively; $K$ is the realized genotypic relationships matrix calculated as the variance–covariance matrix of the principal component analysis from SNP data, following Price et al. (2006), Malosetti et al. (2007a), and Gutiérrez et al. (2011); $\sigma^2_G$ is the genetic variance; $I$ is an identity matrix; and $\sigma^2_e$ is the residual variance. The GWAS was performed with the R package lmer.gwaser (Gutiérrez et al., 2016), with the significance threshold adjusted by the effective number of independent tests (Li and Ji, 2005). The significant SNPs on each chromosome were clustered by their physical positions with the hclust and cutree basic R functions (R Core Team, 2015) with an $h$ parameter of a quarter of the maximum height of the tree. Clusters with three or more SNPs were considered to be a QTL, and the SNP with the highest $-\log_10(P)$ at each QTL was selected. The selected SNPs from QTL for FT and PH were used as covariates for GWAS scans of the disease traits, following von Zitzewitz et al. (2011) and Locatelli et al. (2013). To show the consequences of not correcting for the confounding factors in the GWAS analysis, a naïve model without FT- and PH-associated SNPs as covariates was also fitted. The selected SNPs from QTL for N. oryzae, R. oryzae-sativae, and T. cucumeris were used for multilocus estimation of the proportion of phenotypic variance (PVE) explained by the QTL, and QTL effects, calculated as percentage of the phenotypic mean. Multilocus models were fitted with the R package EMMREML (Akdemir and Godfrey, 2014) following Gutiérrez et al. (2015). Regions of chromosomes 9 and 12 were analyzed for linkage disequilibrium (LD) blocks. Linkage disequilibrium was computed as pairwise $R^2$ between all SNPs in the region, and limits between LD blocks were graphically assessed with the R package LDheatmap (Shin et al., 2006). The physical positions of the QTL described in the literature for sheath blight, PH, and FT were based on the review by Zeng et al. (2015).

RESULTS

Phenotyping

Adjusted phenotypic means of tropical japonica were significantly different ($P < 0.01$) from those of the indica population for all traits. Tropical japonica lines were, on average, taller, later to flower, and more susceptible to stem and sheath diseases than indica lines. Genetic variance and generalized heritability did not differ significantly between populations ($P < 0.01$). Resistance to N. oryzae and R. oryzae-sativae had higher heritability in greenhouse than in field trials (Table 1). Field trials had intermediate to high heritability for resistance to N. oryzae, FT, and PH, and low or very low for noninoculated R. oryzae-sativae experiments. T. cucumeris had lower heritability than N. oryzae and R. oryzae-sativae in greenhouse trials.

Flowering time in tropical japonica field trials was correlated ($P < 0.05$) with the proxy for FT in tropical japonica greenhouse trials, whereas FT in indica field trials was not correlated with FT in indica greenhouse trials (Table 2). Flowering time was not correlated with disease severity in tropical japonica field trials, but it was negatively correlated with disease severity in tropical japonica greenhouse trials (Table 2). Inversely, FT was negatively correlated with disease severity in indica field trials and
was not correlated or had low positive correlations with disease severity in *indica* greenhouse trials (Table 2). Flowering time was negatively correlated with PH in tropical *japonica* and positively correlated with PH in the *indica* population. Resistance to the three diseases correlated positively in tropical *japonica* and *indica* populations. Five additional QTL were found exclusively in tropical *japonica* and 23 in *indica*. Most QTL for resistance to stem and sheath diseases (two in both populations, five in tropical *japonica*, and 22 in *indica*) did not colocalize with QTL for FT or PL, but four disease QTL did colocalize with QTL for FT, PH, or both and were not included in further analyses. Multilocus analysis of QTL for disease resistance revealed major-effect loci with PVE ranging from 10 to 43, with the exception of *R. oryzae-sativae* evaluated in greenhouse trials (Fig. 1).

Favorable alleles at QTL discovered for resistance to *N. oryzae* were also favorable or neutral towards resistance to *R. oryzae-sativae* and vice versa for all trials, environments and populations (Fig. 2). Favorable alleles at QTL for resistance to *N. oryzae* and *R. oryzae-sativae* had little effect on resistance to *T. cucumeris*. Allele substitution effects of QTL for resistance to *T. cucumeris*, *N. oryzae*, and *R. oryzae-sativae* accounted for less than 2% of the phenotypic mean on FT and less than 4% of the phenotypic mean on PH (Fig. 2).

A region on chromosome 9 from 12 to 23 Mb, encompassed multiple QTL for resistance to *N. oryzae*, *R. oryzae-sativae*, and *T. cucumeris* in field and greenhouse trials in tropical *japonica* (Fig. 3) and in *indica* (Fig. 4).

For tropical *japonica*, three out of five major LD blocks

### Table 1. Generalized heritability of field and greenhouse (GH) trials for *N. oryzae*, *R. oryzae-sativae*, sheath blight caused by *T. cucumeris*, plant height (PH), and flowering time (FT) in tropical *japonica* (Trj) and *indica* (Ind) rice populations.

<table>
<thead>
<tr>
<th>Trial</th>
<th><em>N. oryzae</em></th>
<th><em>R. oryzae-sativae</em></th>
<th><em>T. cucumeris</em></th>
<th>PH</th>
<th>FT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field 2009</td>
<td>0.56 (0.06)</td>
<td>–</td>
<td>0.41 (0.06)†</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Field 2010</td>
<td>0.40 (0.08)</td>
<td>–</td>
<td>0.19 (0.15)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Field 2011</td>
<td>0.52 (0.09)</td>
<td>0.32 (0.09)</td>
<td>0.02 (0.17)</td>
<td>0.08 (0.16)</td>
<td>–</td>
</tr>
<tr>
<td>Field 2012</td>
<td>0.60 (0.04)</td>
<td>0.52 (0.06)</td>
<td>0.30 (0.10)</td>
<td>0.10 (0.13)</td>
<td>–</td>
</tr>
<tr>
<td>Field 2013</td>
<td>0.62 (0.02)</td>
<td>0.54 (0.04)</td>
<td>0.43 (0.05)</td>
<td>0.53 (0.02)</td>
<td>–</td>
</tr>
<tr>
<td>GH 1</td>
<td>0.69 (0.04)</td>
<td>0.66 (0.04)</td>
<td>0.79 (0.03)</td>
<td>0.85 (0.04)</td>
<td>0.65 (0.13)</td>
</tr>
<tr>
<td>GH 2</td>
<td>0.75 (0.04)</td>
<td>0.79 (0.06)</td>
<td>0.85 (0.07)</td>
<td>0.76 (0.05)</td>
<td>0.50 (0.12)</td>
</tr>
<tr>
<td>GH 3</td>
<td>–</td>
<td>–</td>
<td>0.80 (0.02)</td>
<td>0.67 (0.04)</td>
<td>–</td>
</tr>
</tbody>
</table>

† The SE of generalized heritability is shown in parentheses.

### Table 2. Pearson’s correlations between weighted phenotypic means of field and greenhouse (GH) trials for *N. oryzae*, *R. oryzae-sativae*, *T. cucumeris*, plant height (PH), and flowering time (FT) in tropical *japonica* (Trj, ordinary typeface) and *indica* (Ind, underlined) rice populations. Only significantly different from zero correlations (P < 0.05) are reported.

<table>
<thead>
<tr>
<th>Ind</th>
<th>Trj</th>
<th>Field</th>
<th>GH</th>
<th>Field</th>
<th>GH</th>
<th>Field</th>
<th>GH</th>
<th>Field</th>
<th>GH</th>
<th>Field</th>
<th>PH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. oryzae</em></td>
<td>Field</td>
<td>–</td>
<td>0.29</td>
<td>0.62</td>
<td>0.13</td>
<td>0.15</td>
<td>NS†</td>
<td>NS</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>0.28</td>
<td>–</td>
<td>0.12</td>
<td>0.26</td>
<td>0.12</td>
<td>0.36</td>
<td>0.30</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. oryzae-sativae</em></td>
<td>Field</td>
<td>0.38</td>
<td>0.24</td>
<td>–</td>
<td>NS</td>
<td>0.20</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>0.37</td>
<td>0.31</td>
<td>0.26</td>
<td>–</td>
<td>NS</td>
<td>0.30</td>
<td>NS</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. cucumeris</em></td>
<td>GH</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-0.13</td>
<td>–</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FT</td>
<td>-0.40</td>
<td>0.30</td>
<td>-0.20</td>
<td>-0.40</td>
<td>NS</td>
<td>–</td>
<td>0.33</td>
<td>-0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH</td>
<td>NS</td>
<td>NS</td>
<td>0.22</td>
<td>0.14</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

† NS, not significantly different from zero.

### Genome-Wide Association Study Scanning and Multilocus Analysis

A major-effect QTL for FT was found in both populations on chromosome 3 (0.5–2.26 Mb in tropical *japonica* and 0.5–2.1 Mb in *indica*), with PVE = 30 in tropical *japonica* and PVE = 24 in *indica*. Plant height was oligogenic in tropical *japonica*, with a major-effect QTL (PVE = 27) on chromosome 1 (34.6–39.8 Mb), and several QTL with PVE < 5 were found for PH in *indica* (Supplemental Fig. S1). When naïve models without covariates were used, several QTL for resistance to the studied diseases colocalized with these FT and PH QTL (Supplemental Fig. S1). The SNPs with highest -log₁₀(P) for FT and PH QTL were used as cofactors in the GWAS scan for resistance to *N. oryzae*, *R. oryzae-sativae*, and *T. cucumeris*. A total of 33 nonoverlapping QTL for resistance to stem and sheath diseases were identified (Fig. 1). The genetic architecture differed for the three diseases in tropical *japonica* and *indica*, with only five QTL identified in the same genomic locations in the two populations. Five additional QTL were found exclusively in tropical *japonica* and 23 in *indica*. Most QTL for resistance to stem and sheath diseases (two in both populations, five in tropical *japonica*, and 22 in *indica*) did not colocalize with QTL for FT or PL, but four disease QTL did colocalize with QTL for FT, PH, or both and were not included in further analyses. Multilocus analysis of QTL for disease resistance revealed major-effect loci with PVE ranging from 10 to 43, with the exception of *R. oryzae-sativae* evaluated in greenhouse trials (Fig. 1).
across the region (Fig. 3, lower section) had SNPs associated with *N. oryzae*, named q9–1, q9–2, and q9–3 (Fig. 3, middle section). The SNP S9_21382492 was associated with resistance to *N. oryzae* in field and greenhouse trials and to *R. oryzae-sativae* in field trials. It was not in LD with the surrounding significant SNPs and was named
Fig. 2. Effects of rice quantitative trait loci (QTL) for resistance to *Nakataea oryzae* (NO), *Rhizoctonia oryzae-sativae* (ROS), and *Thanatephorus cucumeris* (TC) in field and greenhouse (GH) on diseases, flowering time (FT), and plant height (PH) in tropical *japonica* (Trj) and *indica* (Ind) populations, estimated with a multilocus model with all the QTL found in this study for each disease. Allele substitution effects are reported as the percentage of the phenotypic mean. Error bars represent SE.
Fig. 3. Zoom-in view of positions 11.5 to 23 Mb of chromosome 9 in tropical japonica rice. The upper section shows the physical positions of previously reported quantitative trait loci (QTL) for flowering time (FT, purple), plant height (PH, green) and resistance to *Thanatephorus cucumeris* (TC, brown) reviewed by Zeng et al. (2015). The QTL found in our study in this region are marked with gray bars. The middle section shows the zoom-in view of the region with genome-wide association study scan results for *Nakataea oryzae* (NO, red), *Rhizoctonia oryzae-sativae* (ROS, blue), TC (orange), FT (purple), and PH (green) in field (triangles) and greenhouse (circles) trials. The lower section shows the pairwise $R^2$ between all single nucleotide polymorphisms in the region. Average $R^2$ values within and between each linkage disequilibrium block are presented.
Fig. 4. Zoom-in view of positions 11.5 to 23 Mb of chromosome 9 in *indica* rice. The upper section shows the physical positions of previously reported quantitative trait loci (QTL) for flowering time (FT, purple), plant height (PH, green) and resistance to *Thanatephorus cucumeris* (TC, brown) reviewed by Zeng et al. (2015). Quantitative trait loci found in our study in this region in tropical *japonica* (Trj) are marked in dark gray bars, and QTL found in *indica* are marked with light gray bars. The middle section shows the zoom-in view of the region with genome-wide association study scan results for *Nakataea oryzae* (NO, red), *Rhizoctonia oryzae-sativae* (ROS, blue), TC (orange), FT (purple), and PH (green) in field (triangles) and greenhouse (circles) trials. The lower section shows the pairwise \( R^2 \) values between all single nucleotide polymorphisms in the region. Average \( R^2 \) values within and between each linkage disequilibrium block are presented.
The SNPs S9_22275709 and S9_22544543 were in LD and together were named q9–5. The QTL q9–5 was associated with resistance to *N. oryzae* in field and greenhouse trials, with *R. oryzae-sativae* resistance in field trials, and with *T. cucumeris* resistance in greenhouse trials in tropical *japonica*. There were no associations between SNPs and FT or PH in this region of chromosome 9 in tropical *japonica*. The QTL q9–3 and q9–5 did not overlap with any previously reported QTL for FT or PH in the region (Fig. 3, upper section). Together, tropical *japonica* QTL q9–3 and q9–5 had an allele substitution effect of 5.2% of the phenotypic mean (*N. oryzae*, field), 10.2% (*N. oryzae*, greenhouse), 8.9% (*R. oryzae-sativae*, field), 17.0% (*R. oryzae-sativae*, greenhouse), and 6.6 (*T. cucumeris*, greenhouse). The allele substitution effect of these QTL on FT and PH were 0.2% and 0.5%, respectively. In *indica*, the LD pattern in the same region of chromosome 9 (Fig. 4, lower section) suggested a much higher rate of historical recombination and a less well defined LD block structure than that in tropical *japonica*. However, pairwise R² between significant SNPs in *indica* allowed their grouping in QTL that partially overlapped those found for tropical *japonica* (Fig. 4, middle section). Specifically, pairwise R² between significant *indica* SNPs in the region from 17 to 20 Mb differentiated two QTL, named q9–3a and q9–3b (Fig. 4). *Indica* q9–3b colocalized with tropical *japonica* q9–3 and was associated with the same traits. Similarly, *indica* SNP S9_22756593 colocalized with tropical *japonica* q9–5 and was associated with resistance to *T. cucumeris* as well (Fig. 4, middle section). The resistant allele substitution effects of *indica* q9–3b and q9–5, estimated as a percentage of the phenotypic mean, were 17.2% (*N. oryzae*, field), 20.2% (*N. oryzae*, greenhouse), 17.0% (*R. oryzae-sativae*, field), 14.0% (*R. oryzae-sativae*, greenhouse), and 8.1% (*T. cucumeris*, greenhouse). The resistance allele substitution effects of q9–3b and q9–5 were 0.4% of the FT mean and 1.6% of the PH mean. Of all the disease resistance QTL found in chromosome 9 for *indica*, only q9–3a had a SNP associated with PH (P = 0.0004) (Fig. 4, middle section).

**DISCUSSION**

**Quantitative Trait Loci for Resistance to Stem and Sheath Diseases Independent of FT and PH**

In the population used in our study, *indica* lines were shorter with earlier maturity and were still more resistant to *N. oryzae*, *R. oryzae-sativae*, and *T. cucumeris* than tropical *japonica* lines on average. However, when correlations were studied within the *indica* population individually, FT and PH were negatively correlated with disease incidence. This is in concordance with reports on late maturing and tall cultivars being found to be more resistant to *R. oryzae-sativae* and *N. oryzae* than modern early maturing, semidwarf ones (McKenzie et al., 1994).

In our study, variation arising on FT and PH in the evaluation of disease resistance was removed statistically using covariates for adjusted phenotypic mean estimation. However, when the proxy for FT from greenhouse trials was used as a covariate, disease resistance-adjusted means still correlated with FT from field trials (Table 2), and QTL found for greenhouse trial-adjusted means still colocalized with FT QTL when a GWAS naïve model was used (Supplemental Fig. S2). This may correspond in part to different phenological controls of flowering time or a strong genotype × environment interaction for this trait, particularly for *indica* trials, which may make FT measured in greenhouse trials irrelevant as a confounding factor for disease rating. This supported the need for additional corrections using SNPs associated with FT and PH as cofactors in the GWAS analysis. Other developmental and morphological traits not addressed in this work such as tiller number and culm angle have also been reported to affect field evaluation of *T. cucumeris* in some studies (Loan et al., 2004; Pinson et al., 2005). However, these traits tend to be fixed or have little phenotypic variation in adapted or advanced breeding populations, no longer affecting disease evaluation (Eizenga et al., 2013; Li et al., 1995; Wang et al., 2011). Thus the remaining genetic effect estimated in our study after removing FT and PH effects presumably corresponds predominantly to physiological disease resistance mechanisms (i.e., those not driven by phenology or morphology).

The QTL of major effect for FT and PH identified in this study correspond to the HD9 QTL (Lin et al., 2002) and the sd-1 gene (Cho et al., 1994), respectively. HD9 was segregating in our material and was the most significant genetic region explaining phenology in both the tropical *japonica* and *indica* populations. The sd-1 gene segregated only in tropical *japonica*; it was fixed in the semidwarf *indica* germplasm, where PH was under polygenic control. Genome-wide analysis study scans for resistance to the studied stem and sheath disease with naïve models (i.e., without SNPs of major QTL for FT and PH as cofactors) identified QTL that colocalized with these FT and PH major-effect QTL, in accordance with previous studies (Zeng et al., 2015). Nelson et al. (2012) reported that one putative QTL for resistance to sheath blight was eliminated when FT and PH were used as covariates. In our study, when SNPs associated with FT and PH were used as cofactors in the GWAS analysis, the corresponding putative disease resistance QTL were eliminated.

The strategies used in this study to minimize the confounding effects of FT and PH were effective, enabling the discovery of 29 QTL for resistance to stem and sheath diseases. Notably, 17 of these QTL did not overlap with previously reported QTL for FT or PH (Zeng et al., 2015). The multidisease resistance locus, q9–3, which was associated with resistance to *T. cucumeris*, *N. oryzae*, and *R. oryzae-sativae*, was not associated with FT or PH in our population (Fig. 3 and Fig. 4). The only exception to this was QTL q9–3a in *indica*, which had an allele substitution effect of 1.6% of the PH mean. This evidence supports the conclusion that most of the QTL reported here contribute to physiological mechanisms of disease resistance, and SNPs within these QTL will be useful for marker-assisted breeding to improve the levels of resistance to the fungal pathogens
Multiple Disease Resistance

T. cucumeris, N. oryzae, and R. oryzae-sativae, the causal agents of sheath blight, stem rot, and aggregated sheath spot, without significantly affecting either FT or PH.

Quantitative Trait Loci for Multiple Disease Resistance

Because of its many advantages, multiple disease resistance is a highly valuable and relevant breeding objective (Wiesner-Hanks and Nelson, 2016). In our work, all QTL associated with resistance to R. oryzae-sativae, N. oryzae, and T. cucumeris in tropical japonica breeding materials reciprocally increased resistance to the other pathogens (Fig. 3, left panel). A similar result was found for N. oryzae and R. oryzae-sativae in indica (Fig. 3, right panel). Furthermore, nine QTL for resistance to N. oryzae, R. oryzae-sativae, or both on chromosomes 1, 4, 5, 6, 9, 11, and 12 colocalized with reported QTL for resistance to T. cucumeris (Zeng et al., 2015). In particular, the region from 12 to 23 Mb on chromosome 9 was rich in disease resistance QTL in tropical japonica and indica (Fig. 3 and Fig. 4). The QTL q9–3 and q9–5, present in both populations, were associated with resistance to stem and sheath diseases, accounted for high PVE, and colocalized with more than 12 QTL for resistance to T. cucumeris in previous studies (Zeng et al., 2015). This suggests either pleiotropy or that genes for specific responses to each pathogen are physically close together in the mapped regions (linkage). Following Wiesner-Hanks and Nelson (2016), the magnitude of the effects of resistant alleles at q9–3 and q9–5 across diseases fitted scenarios of multiple disease resistance, with pleiotropy for N. oryzae and R. oryzae-sativae, and with slightly uneven pleiotropy for T. cucumeris vs. N. oryzae or R. oryzae-sativae.

CONCLUSIONS

The confounding effect of some phenological and morphological traits on resistance to stem and sheath diseases can be efficiently controlled for by using appropriate phenotyping and analytical methodologies. When the effect of confounding factors is removed, the QTL identified for disease resistance have little effect on FT and PH. The effects of the disease resistance QTL found in this work suggest the existence of common physiological mechanisms for resistance to sheath blight, stem rot, and aggregated sheath spot, three of the main fungal diseases affecting rice in temperate and tropical areas worldwide. Markers associated with these QTL can be applied in marker-assisted breeding strategies to improve resistance to these three diseases in high-yielding elite germplasm without affecting FT and PH.

Supplemental Information Available

Supplemental Fig. S1. Infection types of stem rot caused by Nakataea oryzae (NO) and depiction of the rating scale used in this study for phenotyping resistance to N. oryzae: 0 = no symptoms (not shown); 1 = dark lesions on the outer sheath and stem without lesions, with (i) superficial dark lesions in the outer sheath and (ii) an uncovered stem with no symptoms; 3 = lesions reaching the outer part of the stem, with (i) growing dark lesions in the outer sheath and (ii) small dark superficial lesions in the uncovered stem (red arrow); 5 = lesions inside the stem, nodes, or both with (i) growing dark lesions in the outer sheath showing some white mycelia and (ii) dark lesions inside the stem (red arrow) and node (white arrow); 7 = wilted stems with mycelia or sclerotia inside; 9 = necrotic and lodged stems.

Supplemental Fig. S2. Infection types of aggregated sheath spot caused by Rhizoctonia oryzae-sativae (ROS) and depiction of the rating scale used in this study for phenotyping resistance to R. oryzae-sativae: 0 = no symptoms (not shown); 1 = lesions limited to the lower 25% of the leaf sheath, with the lower sheath showing a characteristic oval lesion with a green center surrounded by a brown margin; 3 = lesions present on lower 50% of the leaf sheath, with (i) tillers showing lesions between 25 and 50% of the leaf sheath, and (ii) characteristic lesions on the lower sheath; 5 = lesions present on more than 50% of the leaf sheath and lower leaves, with (i) tillers showing lesions on more than 50% of the leaf sheath and lower leaves, and (ii) characteristic lesions on higher sheaths; 7 = lesions present on more than 75% of the leaf sheath and the flag leaf affected, with (i) tillers showing lesions on more than 75% and an affected leaf sheath, and (ii) characteristic lesions on wilted sheaths; and 9 = severe infection or necrotic tiller, with (i) tillers showing severe infections and (ii) characteristic lesions on necrotic tillers. The same scale was used for rating resistance to Thanatephorus cucumeris (TC).

Supplemental Fig. S3. Manhattan plots of the GWAS scan for flowering time (FT, purple), plant height (PH, green), resistance to Nakataea oryzae (NO, red), and resistance to Rhizoctonia oryzae-sativae (ROS, blue) in field (triangles) and greenhouse (circles) trials for tropical japonica (left panel) and indica (right panel) populations using the naïve model without covariates for GWAS analysis. Phenotypic disease means were estimated with the naïve model, or with FT and PH covariate correction (corr.). The SNPs in the region of defined QTL are highlighted in orange for PH and in light green for the other traits.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

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