Genome-Wide Associations with Resistance to Bipolaris Leaf Spot (*Bipolaris oryzae* (Breda de Haan) Shoemaker) in a Northern Switchgrass Population (*Panicum virgatum* L.)

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ABSTRACT

Switchgrass (*Panicum virgatum* L.), a northern native perennial grass, suffers from yield reduction from Bipolaris leaf spot caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker. This study aimed for determining the resistant populations via multiple phenotyping approaches and identifying potential resistance genes to the disease from genome-wide association studies in the switchgrass northern association panel. The disease resistance was evaluated from both natural (field evaluations in NY and PA) and artificial inoculations (detached leaf and leaf disk assays). There are ten out of the 66 populations showed the most resistant based on a combination of detached leaf, leaf disk, and mean from two locations. The GWAS from five subgroups from the association panel to different disease evaluation combinations yielded 27 significant SNPs on 12 chromosomes: 1K, 2K, 2N, 3K, 3N, 4N, 5K, 5N, 6N, 7K, 7N, and 9N accumulatively explaining phenotypic variance of BLUPs of detached leaf percent lesion via image analysis 26.52% at most and BLUPs of leaf disk percent lesion via image analysis 3.28% at least. Within linkage disequilibrium of 20 kb, these SNP markers linked with the potential resistance genes including genes encoding for NBS-LRR, PPR, cell wall related proteins, homeostatic proteins, anti-apoptotic proteins, and ABC transporters.

INTRODUCTION

Switchgrass (*Panicum virgatum* L.) is a perennial biomass crop native to North America. Biomass and other agronomic traits have been the focus of breeding aims (Godshalk et al., 1998; Hopkins et al., 1993; Talbert et al., 1983; Vogel et al., 2002). Although many diseases have been reported to cause deleterious effects on yield, research on disease resistance, especially breeding for the resistance, is scarce. Only 28 of the 1693 research articles were directly related to diseases in switchgrass based on AGRICultural OnLine Access (AGRICOLA) (2018). *Bipolaris oryzae* (Breda de Haan) Shoemaker (teleomorph: *Cochliobolus miyabeanus*) is one of the major fungi causing Bipolaris leaf spot (BLS) in switchgrass that can reduce biomass by 70% (Fajolu, 2012). To manage the disease, breeding is one of the most economical approaches (Casler, 2012).

The natural distribution of switchgrass is broad latitudes across North America east of the Rocky Mountains. By simple morphological differentiation, switchgrass can be separated into two groups of upland and lowland ecotypes. Upland ecotypes are well adapted to higher latitudes and have higher drought tolerance whereas lowland ecotypes provide higher yield and require more water (Stroup et al., 2003). The more in-depth genetic diversity was revealed by Network-Based single nucleotide polymorphism (SNP) discovery protocol. It suggested the differentiation of switchgrass groups by isolation-by-ploidy, the migration from south to north, and the incidence of tetraploid upland from octaploid upland (Lu et al., 2013). Such a large diversity of switchgrass populations can provide a source for disease resistance (Hoisington et al., 1999).

Plant-pathogen interactions are complicated. The basal defense is initiated when conserved molecular signatures of pathogens called pathogen-associated molecular patterns

70 (PAMPs) are recognized by plant pattern recognition receptors (PRRs). Such a recognition
71 activate PAMP-triggered immunity (PTI). Successful pathogens can suppress PTI by
72 secreting virulent effector proteins. These effectors then trigger the second line of defense
73 called effector-triggered immunity (ETI) mediated by resistance R genes (Jones & Dangl,
74 2006). In biothrophic pathogens, a gene-for-gene interaction between resistance R genes of
75 the plant and avirulent (Avr) genes of the pathogen result in a hypersensitive response leading
76 to local programmed cell death and and the end of colonization, confering resistance. Since *B.*
77 *oryzae* is a necrotrophic pathogen (Ou, 1985), the interaction between switchgrass and the
78 fungus was modelled potentially as an inverse gene-for-gene model (Friesen et al., 2007). In
79 general, the pathogen secretes necrotrophic effectors as host-selective toxins (HST), such as
80 SnTox1 from *Stagonospora nodorum*, T-toxin from *Cochliobolus heterostrophus*, and HC-
81 toxin from *C. carbonum* (Liu et al., 2004; Ullstrup, 1972; Walton, 1996), that interact with
82 host sensitivity genes, resulting in a compatible susceptible interaction to trigger host cell
83 death (Friesen et al., 2008). Such an interaction is known as effector-triggered susceptibility
84 (ETS). Although crude extract from *B. oryzae* was proposed to contain HST (Vidhyasekaran
85 et al., 1986), the toxin has never been characterized. Instead, according to comparative ge-
86 nome study (Condon et al., 2013) and screening on a wide range of hosts (Chakrabarti, 2001;
87 De Bruyne et al., 2016), *B. oryzae* does not produce any HST but produces ophiobolin A and
88 B as a non-host-selective toxin (Xue et al., 2015) triggering many pathways such as reactive
89 oxygen species (ROS) detoxification, protein phosphorylation, and ethylene production (Kim
90 et al., 2014; Bockhaven et al., 2015).

Such an ETS interaction suggested that susceptible cultivars can be rapidly screened for pathogens carrying the necrotrophic effectors (Juliana et al., 2018). Breeding for the improvement of resistance to BLS can be done by eliminating susceptible alleles from the population. However, the recurrent phenotypic selection for the resistance in upland switchgrass cultivars 'Shelter' and 'Cave-in-Rock' for two cycles of selection did not provide any more resistance to BLS (Songsomboon, 2019). The screening was done in a seedling stage; therefore, future breeding for the resistance with various screening methods, in various growth stages, and bigger plant populations was suggested. Identification of resistant genotypes or populations is still needed.

To accelerate breeding for disease resistance, genomics-assisted breeding is an important approach (Leng et al., 2017). Basically, it begins with gene identification, isolation, cloning, functional characterization, validation and utilization. There are two main approaches to identify resistance genes in a diverse population: linkage mapping and genome-wide association studies (GWAS). Although GWAS cannot confirm the causal polymorphism, it depends on linkage disequilibrium (LD) that potentially link between the markers and the causal polymorphisms. The diversity panel in GWAS contains high allelic diversity and ancestral recombination events resulting in a finer resolution than linkage mapping (Yu and Buckler, 2006). The technique has been used to dissect flowering time in switchgrass (Grabowski et al., 2017). Although GWAS has never been used to dissect disease resistance in switchgrass, it has successfully dissected resistance to leaf rust caused by *Puccinia triticina* Eriks., tan spot caused by *Pyrenophora tritici-repentis* (Die.) Shoemaker and stripe rust caused by *Puccinia striiformis* West. in wheat (*Triticum aestivum* L.) (Juliana et al., 2018). Despite no resistance genes identified in switchgrass, there were 13 SNP markers from quantitative trait loci

114 (QTLs) linked with the resistance in rice (Sato et al., 2008, 2015). This study will provide the
115 first dissection of the resistance to BLS in switchgrass.
116 The objectives were 1) to determine genotypes or populations from the northern
117 switchgrass association panel that can be candidates for resistance to BLS, 2) to conduct a
118 single- and multi-trait GWAS for resistance to BLS in the association panel via both natural
119 and artificial inoculation, and 3) to explore the genes linked to the significant markers from
120 the GWAS model to determine potential candidate genes for resistance to BLS.
121

122 **MATERIALS AND METHODS**

123

124 **Switchgrass in the Northern Association Panel**

125 The switchgrass population used in this study had been developed for accelerating
126 breeding progress especially for bioenergy traits at northern latitudes (Lu et al., 2013; Lipka et
127 al., 2014). The association panel consists of 478 plant genotypes from 66 populations repre-
128 senting mostly the upland northern population and some southern lowland populations. 'Pop-
129 ulation' in this study means a seed source with a specific origin and, in some cases, breeding
130 history. Six to ten seeds from each population were planted in to individual plants called
131 "genotypes". The association panel was initially planted in Ithaca, NY, in 2008 and then vege-
132 tatively cloned and planted in a randomized, complete block design with three replicates in
133 0.9 m^2 spaced planting in Ithaca, NY, and Philipsburg, PA, in 2016. No fungicide had been
134 applied to the field.
135
136

137 **Disease evaluation and phenotype processing**

138 Since the unsuccessful recurrent phenotypic selection for resistance to BLS was done
139 in the seedling stage (Songsomboon, 2019), the phenotypic evaluations in this study were ex-
140 plored at a mature stage and different conditions. Bipolaris leaf spot has been evaluated in
141 both fields to determine the resistance from natural incidence under different environments
142 and laboratory to minimize environmental effects on the resistance. Field evaluation was con-
143 ducted in the same week in both locations in August 2017. Each plant was visually evaluated
144 by one person to reduce the variance among persons. The disease severity score ranged from
145 0 to 5, as 0 = 0% BLS, 1= 1-10% BLS, 2= 11-25% BLS, 3= 26-50% 134 BLS, 4= 51-75%
146 BLS, and 5= 76-100% of the leaf area of the whole plant with BLS. In addition to field evalu-
147 ation, to minimize environmental effects, an artificial inoculation in the laboratory was con-
148 ducted. Disease severity was evaluated by detached leaf and leaf disk assays by taking leaf
149 samples from one replicate of genotypes in Ithaca, NY. In the detached leaf assay, three
150 healthy leaves were randomly selected from 30 centimeters below the top of the shoot to con-
151 trol the same stage of leaf development. The leaves were cut into five centimeter segments,
152 washed with deionized water, placed in a pre-wet petri dish bedded with filter paper, and kept
153 cold in a cooler in the field, then refrigerated overnight.

154 The inoculum was prepared from a subculture of a single conidium isolated from a
155 'Carthage' switchgrass leaf in the warm season biofuels field experiment in Ithaca, NY (Wax-
156 man, 2011). The day after collecting the detached leaf, the three-week-old plate was flooded,
157 filtered via gauze, and the concentration adjusted to 10^5 conidia·ml^{-1} by hemocytometer with 2
158 drops of Tween-20 per 100 ml. An airbrush pressuring at 10 psi was used to spray inoculum

159 on each dry-surfaced adaxial side of the leaf in each plate. After letting the droplets of inocu-
160 lum dry and firmly attach to the leaf surface, the plates were then sealed with a paraffin tape
161 to maintain high moisture condition. Each plate contained three detached leaves from one
162 genotype. The plates were placed on table randomly and kept at room temperature with 12-
163 hour light for seven days showing necrotic lesions and leaf necrosis (Figure 1). Due to the
164 limitation of laboratory space for the plates and labor in the leaf collection, 85 to 200 geno-
165 types were sampled in each set of the experiment, with some overlapping genotypes in one to
166 three duplicated plates and additional non-inoculated plates as controls. The leaf samplings
167 were done weekly from May to June 2017 but were controlled by the attempt to sample the
168 similar stage of leaf by sampling from the same height from the top. A total of seven sets of
169 the experiment eventually covered all of 478 genotypes. The disease evaluation was
170 conducted by vision and by image analysis software. Both evaluations were based on
171 percentage of leaf covered with lesions. The evaluation by vision was from 0 to 100 % by
172 10% increments. Then, each leaf was dried and scanned with a flatbed scanner (Canon Ca-
173 noScan LiDE 700F) at a resolution of 1,200 dpi, and images were saved in .tiff format. Alt-
174 hough the detached leaf assay was an unbalanced design, best linear unbiased predictions
175 (BLUPs) to extract random effect of the genotypes was suggested to be able to alleviate the
176 problem by fitting the experiment factor as a fixed effect (Hill & Rosenberger, 1985; Piepho
177 et al., 2008; Robinson, 1991; Stroup & Mulitze 1991; White & Hodge, 2013).
178 In the leaf disk evaluation, all of 478 genotypes were collected on the same day in Au-
179 gust 2017 by cutting the same age leaves from each genotype and keeping them refrigerated
180 overnight. On the next day, the leaves were washed with deionized water, surface dried, bored
181 by keeping the midrib in the middle into an 8-mm disk, and placed on a water agar plate.

182 There were 28 leaf disks in each plate, including four check disks (non-inoculated control and
183 inoculated disks of resistant 'SW43_09' and susceptible 'SW122_02') and three replicated
184 disks of eight genotypes (Figure 2). The inoculum was prepared the same way described
185 above. For each leaf, two μL of inoculum was dropped in the middle of the right side of the
186 leaf. After letting the droplet dry, the total of 64 plates were sealed with paraffin tape to main-
187 tain high moisture, randomly placed on a table, and kept under 12-hour light at room tempera-
188 ture. The disease evaluation was conducted at seven dpi both by vision and image analysis
189 software. The percent of leaf disk covered with lesions and necrotic tissue was assessed by
190 vision from 0% to 100% with 10% increments. Also, on the same day, a photo was taken of
191 each plate with a digital DSLR Canon Rebel T6 DSLR camera with the resolution of 2644 x
192 3084 in .JPG.

193 Images from both detached leaf and leaf disk assays were analyzed by using ImageJ
194 macro at the setting provided in the Supplement file. In brief, each detached leaf or leaf disk
195 was measured for total leaf area and total necrosis lesion area (yellow and black area). Per-
196 centage of area covered with lesions was computed by dividing the total necrotic lesion area
197 by total leaf area.

198 Package 'LME4' (Bates et al., 2015) in R was used to calculate BLUPs from various
199 disease evaluations. For the field evaluation, BLUPs were computed based on each location
200 separately and on the two locations combined using genotypes nested in populations and rep-
201 licates as random effects in each location model and populations as a fixed effect. BLUPs for
202 the two locations combined were fitted as genotype nested in population, replicates, locations,
203 and the interaction between genotypes and location as random effects and populations as a
204 fixed effect. In addition to generating BLUPs from the field evaluations, the highest score,

205 which is the most severe symptoms observed from each location and the combined two loca-
206 tions over replicates, were used based on the successful example of QTLs of resistance to fo-
207 liar symptoms caused by potato virus Y in autotetraploid potato (*Solanum tuberosum* L.)
208 (Silva et al., 2017). The highest scores were used on the assumption that in the field there was
209 a chance that some replicates may be exposed by or avoid the pathogens differently. The max-
210 imum scores were considered the worst response of each genotype. For disease evaluations
211 under laboratory condition, since all phenotypes were expressed as percentages to leaf area,
212 log transformation was performed before computing BLUPs. For the detached leaf assay,
213 BLUPs were computed with experiment, genotype nested in populations, interaction between
214 genotypes and experiment, plates within the experiment, and leaf replicates of genotypes as
215 random effects and populations as a fixed effect. For the leaf disk assay, BLUPs were calcu-
216 lated with plates as a fixed effect and genotype, replicates within plates, and genotype within
217 plates as random effects and populations as a fixed effect. Broad-sense heritability was
218 estimated from variances in each model and standard error was computed by bootstrap ap-
219 proach for 1000 times (Supplement file). Moreover, phenotypic correlations were computed
220 between resistance to Bipolaris leaf spot to the 20 morphological and biomass quality traits
221 from Lipka et al. (2014), such as plant height, anthesis date, acid detergent lignin, minerals,
222 ethanol·g^{-1} dry weight, etc.
223 Therefore, the resistance to BLS was evaluated via three approaches – detached leaf,
224 leaf disk, and field evaluation – providing phenotyped traits including BLUPs of detached leaf
225 percent lesion via vision and image analysis (DTVI and DTIA), BLUPs of leaf disk percent
226 lesion via vision and image analysis (DSVI and DSIA), BLUPs of field evaluation from two

227 locations, NY and PA (BTL, BNY, and BPA), highest score from two locations, NY and PA
228 (MTL, MNY, and MPA).
229

230 **Genotyping, linkage disequilibrium analysis, and population structure**

231 HAPMAP v.1 set from Evans et al. (2015) provided 1,377,841single-nucleotide poly-
232 morphisms covering 38,654 genic regions. In short, DNA of each genotype was processed via
233 exome-capture using the Roch-Nimblegen switchgrass exome-capture probe set (Evans et al.,
234 2014) and DNA sequencing. The sequences were aligned to the *P. virgatum* genome assembly
235 v.4.1 (*P. virgatum* v.4.1, DOE-JGI, http://phytozome.jgi.doe.gov/) for SNP discovery (Ram-
236 stein et al., 2018). The reads were sorted with PicardTools version 2.1.1 (http://broadinsti-
237 tute.github.io/picard) SortSam and MarkDuplicates. Samtools version 1.3.1 (Li et al., 2009)
238 was used to pile up files with base alignment quality disabled and map quality adjustment dis-
239 abled. In this calling, SNPs were required to be bi-allelic, sequenced in all samples, be mono-
240 morphic in at least two samples, filtered a minimum read mapping quality score of 30, and
241 more than 5X coverage in more than 95% of the samples. At each SNP, the genotype dosages
242 ranged from zero to two copies of minor alleles and can be nonintegers by using EM algo-
243 rithm (Martin et al., 2010). Naturally, the switchgrass association panel includes allopolyploid
244 switchgrass: tetraploids (4x), octoploids (8x) and hexaploids (6x). It is challenging to perform
245 GWAS with polyploid models. In this study, we modelled them under the assumption of diso-
246 mic inheritance similar to the study of GWAS of flowering time in Grabowski et al. (2017).
247 The reason was that tetraploid switchgrass was confirmed to show disomic inheritance
248 (Okada et al., 2010). Although octaploid switchgrass had four copies of each homologous
249 chromosome, which was difficult to precisely determine heterozygous genotypes, the disomic

250 segregation was used for the model with the caution of the increasing standard error of esti-
251 mates in GWAS. Moreover, linkage disequilibrium (LD) was computed via '-r2' tag in
252 PLINK as r^2 between all SNPs within 1 MB and recorded all values (Purcell et al., 2007).
253 Principle component analysis was used to evaluate the population structure via PCA in Fac-
254 toMineR package (Lê & Husson, 2008). Additionally, ADMIXTURE was used to evaluate
255 population structure with five-fold cross-validation (Alexander et al., 2009).
256 We were also interested in testing whether the variations in phenotypes were from the
257 high differentiation across five ecotypes (lowland north, lowland south, upland east, upland
258 north, upland west) based on Qst-Fst analysis. Estimated Qst-Fsts were computed by R pack-
259 age "QstFstComp" by bootstrapping 1,000 times by sampling random genotypes (Gilbert &
260 Whitlock, 2015).
261
262 **Genome-wide association studies**
263 Genome-wide Efficient Mixed Model Association (GEMMA) (Zhou and Stephens,
264 2012) was used to implement a multivariate linear mixed model for GWAS of single- and
265 multiple-phenotyping approaches for resistance to BLS, to analyze each phenotype and com-
266 bined phenotypes for improving statistical power (Porter & O'Reilly, 2017; Zhu et al., 2015).
267 Kinship was included as a random effect. Also, the first three principal components (PCs)
268 were included as fixed covariates. Although the results of Bayesian information criterion re-
269 sulted in no improvement of the GWAS model with PC inclusion (Lipka et al., 2013), the in-
270 clusion of PCs helped to improve the QQ-plot closer to the theoretical QQ-plot for this re-
271 sistance to BLS. The GWAS was conducted in five groups including all of 478, tetraploid
272 (4X), octaploid (8X), lowland, and upland genotypes to determine if there were any SNPs

273 linked to specific switchgrass populations. Minor allele frequency (MAF) at 0.05 was used to
274 filter the minor alleles. In total, 135,791 SNP markers were used in the association analysis.
275 To correct multiple testing, false discovery rate (FDR) was calculated as $\frac{\frac{P}{k}(1-\frac{A}{T})}{\frac{A}{T}(1-\frac{P}{k})}$ where P is the
276 p-value tested at 0.1, k is number of traits in the combination, A is the number of SNPs that
277 were significant at the p-value tested and T is the total number of SNPs tested. The significant
278 markers then were used to determine candidate genes linked to them within the range of LD
279 and search in JBrowse in *P. virgatum* v.4.1 in Phytozome (e.g., if the position of the marker
280 was 4019500 on chromosome 9K and LD was 20 kb, "Chr09K:4019500..4039500" was
281 used). In case that the candidate gene was not fully aligned within that LD range, we consid-
282 ered the gene as a candidate if 80% of its length was within the LD range. Since some of
283 SNPs' positions could not be aligned to the *P. virgatum* genome assembly v.4.1, they were
284 excluded from determining the candidate genes.
285
286 **Candidate genes based on resistance in rice**
287 Since *B. oryzae* is one of the major pathogens in rice (*Oryza sativa*), major resistance
288 QTLs have been identified in recombinant inbred lines (RILs) and double haploids (DH) by
289 using restriction fragment length polymorphism (RFLP), simple-sequence repeat (SSR)
290 markers, and sequence- tagged site (STS) (Sato et al., 2008, 2015; Katara et al., 2010). Three
291 major QTLs in Chromosomes 1, 4 and 11 were then studied further in near-isogenic lines
292 (NILs) and 13 SNP markers linked with the resistance (Sato et al., 2015). The NILs are
293 BC3F5 between indica 'Tadukan' (resistance) and temperate japonica 'Koshihikari' (suscepti-
294 ble). According to GWAS in rice, linkage disequilibrium (LD) are ~ 100 kb in indica and

295 ~200 kb temperate in japonica (Zhao et al., 2011). Thus, in this study, the candidate resistance
296 genes were screened from 200 kb around the 13 SNPs from *Oryza sativa* genome assembly
297 v.7.0 (*Oryza sativa* v.7.0, DOE-JGI, http://phytozome.jgi.doe.gov/). The candidate genes
298 from rice are listed in the table S1. The sequences of these candidate genes were used to iden-
299 tify potential homologs in switchgrass by using the BLASTP tool on Phytozome and kept the
300 hits with top 10% highest score (Goodstein et al., 2012).
301
302 **RESULTS**
303
304 **Phenotyping and correlation between traits**
305 The results from detached leaf showed that 'ECS.6' had the lowest severity (DTVI
306 24% and DTIA 8%) and 'SW115' had the highest severity among populations (DTVI 81%
307 and DTIA 60%) (Table S2). However, they were not significantly different from the other
308 population with lowest and highest severities (Table Stat1&2). When comparing among geno-
309 types, 'SW788.05' had the lowest severity (DTVI 6% and DTIA 2%) whereas 'SW63.05' had
310 the highest severity (DTVI 100% and DTIA 100%) (Table S3). Similarly, they were not sig-
311 nificantly different from the other genotypes with lowest and highest severities (Table
312 Stat11&12). The leaf disk assay showed that 'SW803' had the lowest severity (DSVI 20%
313 and DSIA 48%) and 'SW38' had the highest severity (DSVI 98% and DSIA 99%) but not sig-
314 nificantly different than other populations (Table Stat3&4). In the genotype-based compari-
315 son, 'High Tide.02' had the lowest severity (DSVI 10% and DSIA 11%) but not significantly
316 different than other genotypes (Table Stat13&14), and the highest severity at DSVI 100% and
317 DSIA 100% among the 81 genotypes. Based on artificial inoculations, both the detached leaf

318 and the leaf disk assay suggested that the lowland ecotypes (DTVI 43%, DTIA 21%, DSVI
319 43% and DSIA 70%) appeared to be more resistant than the upland ecotypes (DTVI 61%,
320 DTIA 39%, DSVI 67% and DSIA 89%) which are statistically significant (p-value < 0.05)
321 (Table S4).

322 In the field evaluation based on the mean of the severity score (0 to 5) from two loca-
323 tions, 'Shelter' showed the lowest severity (0.44) and 'SW787' the highest severity but not
324 significantly different from the other populations (Table Stat5). However, when considering
325 each location, different populations performed differently. In comparison between NY and
326 PA, Wilcoxson's rank test showed significant difference between the two locations (p-
327 value=0.042). The re-ranking incidence (Le et al., 2001; Muir et al., 1992) across locations
328 indicates the significant GxE effect. In NY, SW803 had the lowest severity (0.22) and SW787
329 had the highest severity (3.0) despite not significantly different from other populations (Table
330 Stat6). Whereas, in PA, Shelter had the lowest severity (0.33) and Pathfinder had the highest
331 severity (2.9) despite not significantly different from other populations (Table Stat9). Between
332 the two locations, 'SW123', 'SW33', 'SW793', 'SW781', High Tide, and Timber had the
333 lowest MTL at 3 (resistant), and 41 populations had the highest MTL at 5 (susceptible) even
334 being not significantly different from the other 66 populations (Table Stat6). In NY, SW123,
335 SW128 and 'ECS.6' had the lowest MNY at 2, and 32 populations had the highest MNY at 5.
336 In PA, 'SW115', 'SW802', 'SW31' and 'SW43' had the lowest MPA at 2, and 19 populations
337 had the highest MPA at 5 without significant differences (Table Stat8&10). Although, each
338 phenotyping approach cannot determine single resistant population, a combination of de-
339 tached leaf, leaf disk, and mean from two locations showed that 'SW788', 'SW806',

340 ‘SW802’, ‘SW793’, ‘SW781’, ‘SW797’, ‘SW798’, ‘SW803’, ‘SW795’, ‘SW805’ were al-
341 ways in the lowest severity group and potential candidates of resistance.
342 When comparing among ecotypes, the field evaluation of means between two locations
343 showed the trend of more resistance in lowland than upland ecotypes significantly (Figure S1
344 & Table S4). The same difference also occurred in NY and PA. The severities based on geno-
345 types in each phenotyping approach are listed in Table S3 and Stat15-18.
346 Broad-sense heritabilities (H^2) in different groups of genotypes were similar across
347 groups with some variations (Table 1). For example, H^2 from DSIA in upland was only 0.14
348 while other groups had around 0.40. Although each trait from different phenotyping ap-
349 proaches in different groups had significantly moderate to high H^2, H^2 of severity from the two
350 locations combined was zero. This was supported by the significantly different mean of sever-
351 ity from the two locations, NY and PA (Figure S2 and Wilcoxson p-value = 0.042). Due to
352 zero heritability, the BTL was zero and cannot be used for conducting GWAS. The different
353 ranking was not only present in the field evaluation, the other phenotyping approaches yielded
354 different ranking of resistance as suggested by the ranks of DTIA, DSIA and mean from two
355 locations and the correlations between the traits (Figure S2, Figure 3 and Wilcoxson’s p-
356 value<2.2e-16). For example, from 478 genotypes, ‘KY1625_07’ ranked 27 from DTIA but
357 ranked 329 in DSIA, and 100 in mean from two locations. Such differences led to low corre-
358 lation among approaches such that the r^2 between DTIA and DSIA was only 0.1 and between
359 DTIA and mean of two locations (TL) was 0.03 (Figure 3). In contrast to these low correla-
360 tions, there were high correlations in DTVI-DTIA (r^2 = 0.84) and DSVI-DSIA (r^2 = 0.85).
361 There was no BLS resistance trait correlating to other agronomic or biomass quality traits
362 (Figure S3).

363 In addition to comparing solely on phenotype, Qst-Fst comparisons were used to in-
364 vestigate whether the mean difference of each of the phenotypes among populations is greater
365 than expected mean difference under genetic drift. None of the traits (DTVI, DTIA, DSVI,
366 and DSIA) has Qst-Fst significantly more than zero (Figure 4 and Figure S4) showing no pos-
367 itive selection for resistance to BLS in populations.
368

369 **Linkage disequilibrium, principal component analysis and admixture**

370 To determine the interval that potentially linked to the significant markers from
371 GWAS, linkage disequilibrium (LD) decay was estimated by plotting the allele frequency cor-
372 relations (R^2) against the physical distance in base pairs (Figure 5). In the full set of 478 geno-
373 types, the LD decayed sharply within 20 kb and reached a plateau background within 50 kb.
374 Therefore, in this study, we focused on genes within the 20 kb from the significant SNPs.
375 Based on the principal component analysis and admixture, there was a population
376 structure in this association panel (Figure 6). From PC1 and PC2, upland and lowland eco-
377 types separated from each group. Also, within the lowland ecotype, the latitude of lowland
378 can be distinguished to lowland north and south groups. The separation of upland ecotype can
379 also be noticed in PC1 and PC3 in that the upland north was grouped apart from the other up-
380 land. Besides ecotypes, ploidy level can be differentiated into groups. Both lowland north and
381 south, and upland north genotypes were tetraploid (4X) while the upland east and upland west
382 genotypes were octaploid (8X). In total, PC1, PC2, and PC3 explained the variance due to the
383 population of 49.35%. The admixture (Figure 5b) also showed the distinction among five
384 groups of ecotypes.
385

386 **Genome-wide association**

387 As we conducted GWAS with five subsets of genotypes including478, 4X, 8X, low-
388 land and upland, most of the single phenotypes did not show significant Manhattan peaks or
389 theoretically-expected QQ-plots (Figure S5 to S8). For multiple combinations of assessing re-
390 sistance to BLS via many phenotyping approaches, almost all the combinations for GWAS
391 did not provide significant peaks or QQ-plots (Supplement file). To improve the association
392 analysis, the GWASs were then conducted with the combination of three traits of DTVI,
393 DTIA and DSIA, which yielded 14 significant peaks passing FDR threshold and the QQ-plot
394 with the expected distribution (Figure 7a and Table 2).

395 In a tetraploid group, the two-trait combination of DSVI and MNY provided three sig-
396 nificant peaks with expected QQ-plot (Figure 7b). Interestingly, one peak of SNP
397 Chr07N_61547213 on chromosome 7N from the tetraploid group overlapped with the same
398 peak in GWAS from the 478 group. In the octaploid group, the two-trait combination of
399 DTVI-DSIA showed one significant peak on chromosome 9N (Figure 7c). In the lowland
400 group, the best GWAS was from the single trait mean of the combined two locations yielding
401 eight peaks (Figure 7d). In the upland group, the combination of DTIA-MTL gave two most
402 significant peaks on chromosome 1k and 7K (Figure 7e).

403 In this study, therefore, we focused on five selected GWAS analyses including 478-
404 DTVI-DTIA-DSIA, 4X-DSVI-MNY, 8X-DTVI-DSIA, low-mean TL, and up-DTIA-MTL
405 (Figure 7). In total, there were 27 significant peaks (one overlap between 478 and lowland
406 group) across 12 chromosomes: 1K, 2K, 2N, 3K, 3N, 4N, 5K, 5N, 6N, 7K, 7N, and 9N. Ac-
407 cumulatively, when considering phenotypic variance explained (PVE) by all 27 significant

408 SNP markers for each trait, PVE of DSIA was the highest at 26.52% and PVE of DTIA was
409 the lowest at 3.28% (Table 3).

410 In 478-DTVI-DTIA-DSIA, the significant markers were on chromosomes 2K, 2N, 3K,
411 4N, 5K, 7K, 7N and 9N. These 14 SNP markers explained phenotypic variances for DTVI,
412 DTIA and DSIA of 4.25, 1.77, and 22.62%, respectively (Table S5). In 4X-DSVI-MNY, there
413 were three SNPs on chromosome 3K, 3N and 7N, accumulatively explaining 9.78% for DSVI
414 and 11.31% for MNY. In 8X-DTVI-DSIA, only one significant marker showed on chromo-
415 some 9N explaining 0.64% of DTVI and 11.31% of MNY. In low-mean TL, the eight signifi-
416 cant markers were on chromosomes 1K, 5N, 6N, 7N, and 9N explaining 45% of mean TL. In
417 up-DTIA-MTL, the significant markers were on chromosome 1K and 7K explaining 5.03% of
418 DTIA and 7.77% of MTL. The PVEs of each SNP in each subgroup and each trait are re-
419 ported in Table S6.

420 In addition to the effect size of each SNP to each trait via PVE, the direction and in-
421 heritance of genotypic effects was shown in the format of allele combination effect of each
422 SNP on phenotypes (Figure 8 and S10-S17). Expectedly, none of the SNPs showed inher-
423 itance with all of phenotypes, but with only some phenotypes. Most of inheritance patterns
424 were dominant (Figure 8a, b, c, d, f, g, m, n, o, and q). A few were over dominant (Figure 8e,
425 h, j, and k). Only Chr07N.48546809 showed and additive effects to DSIA as it contributes to
426 the trait with PVE at 2.31 (Figure 8i). However, the PVE did not explain PVE. For example,
427 Chr07N.61547213 had PVE 3.67 for MNY but did not show inheritance (Figure 8p).

428 When comparing these significant SNP markers to the potential candidate genes for
429 resistance to BLS in rice, Chr07N_48546809, on chromosome 7N from 478-DTVI-DTIA-

430 DSIA linking with Pavir.7NG240300 overlapped with LOC_Os04g39430.1 on rice chromo-
431 some 4 from AE11005627 marker (Table S1). Moreover, Chr07_54502620, on chromosome
432 7K from up-DTIA-MTL linking with Pavir.7KG238800 overlapped with LOC_Os04g43730
433 on rice chromosome 4 from ad04009558 marker.
434

435 **DISCUSSION**

436 **The most resistant populations for improvement of resistance to BLS**

437 Since the various phenotyping approaches yielded various resistant populations, it was
438 challenging to determine the most resistant candidates for further breeding. Based on field
439 evaluation, BLUPs from two locations were zero due to zero broad-sense heritability. The dif-
440 ferent trend for BLS resistance between two locations was confirmed by the significant rank
441 test. This re-ranking incidence suggested that the resistance was under GxE effects between
442 two locations (Le et al., 2001; Muir et al., 1992). If the mean of field scores across the two lo-
443 cations were used, the upland Shelter should be considered the most resistant population
444 across two locations with a mean of 0.44. The high resistance of Shelter from the field evalua-
445 tions could explain the result in a prior study (Songsomboon, 2019) that it cannot be improved
446 for resistance to BLS via recurrent phenotypic selection in seedlings. However, the one of
447 most susceptible SW787 had the mean score only 2.33. This suggested the low natural inocu-
448 lation in 2017, which was not conducive to severe disease development. When comparing
449 each location, SW803 showed lowest severity in NY but not significantly different than the
450 other 55 genotypes, but it had highest score in PA at 2.11, which was significantly different.
451 Therefore, this variation needs to be confirmed with more replicates, years, and locations. One
452 more location in New Jersey was transplanted in 2018.

To lessen the GxE effect on the BLS, artificial inoculation was conducted in the laboratory. In the detached leaf assay, due to the limitation of sampling, a whole set of 478 genotypes cannot be tested at the same time. The experiment was separated into seven overlapping sets. However, there was a significant variation among sets. Such a variation can occur from the different age of each genotype in the same sampling time. Although the same stage of leaves was sampled at 30 centimeters from the top, variation existed among leaves from the same plant. To handle the variation among sets and unbalanced design (Hill & Rosenberger, 1985; Piepho et al., 2008; Robinson, 1991; Stroup & Mulitze 1991; White & Hodge, 2013), BLUPs were used by fitting the effect from the set as a fixed effect. Another artificial inoculation was leaf disk assay, with less limitation of the experimental size, the whole set of 478 genotypes was sampled at the same time with resistant and susceptible checks in each plate. However, this experiment was conducted only once at the end of growing season. Although a small effect of plates existed, BLUPs was fitted by considering plates as a fixed effect. Both artificial inoculations were evaluated via vision and image analysis. The image analysis is better regarding repeatability and reanalysis in the future (Stewart and McDonald, 2014). For the sake of comparison for the most resistant population, mean of percent lesion from image analysis was considered. In the detached leaf approach, the lowest severity population from DTIA was ECS.6 at 8% (not significantly different than the other 26 populations), but the population appeared very susceptible from DSIA at 94%. In the leaf disk assay, the most resistant population from DSIA was SW803 with 20 other populations. These supported the low correlation among all three disease evaluation approaches. The low correlation between laboratory and field evaluations could be explained by the different disease pressure between the two conditions. The natural inoculation in the field varied by the weather condition of each year

476 whereas the laboratory inoculation was conducted with the high inoculum concentration to
477 maximize the disease pressure. Another concern was the low correlation between detached
478 leaf assay and leaf disk assay. It can be the result of the different ratio of leaf area to inocu-
479 lum. The detached leaf assay used 5-cm long whole leaf and spraying inoculum whereas the
480 leaf disk assay used only 8-mm bored leaf section with a 2-microliter inoculum droplet on a
481 single location. The difference between spraying and droplet also showed in the assessment of
482 early blight (*Alternaria solani*) (Chaerani et al., 2017).

483 The Qst-Fst of all phenotypes showed that none of them has gone under positive selec-
484 tion across five ecotypes. This neutrality was not expected as most of the resistance genes
485 were hypothesized to be under natural selection (Grant et al. 1995, 1998; Stahl et al. 1999;
486 Tian et al. 2002, 2003). However, neutral evolution of resistance genes like nucleotide bind-
487 ing sites – leucine rice repeats (NBS-LRR) can be undergone in a relaxed selective constraint.
488 For example, when pathogens are absent or at low frequency, the resistance genes may expe-
489 rience much weaker or no cost of resistance (Gos and Wright, 2008). Such a low disease pres-
490 sure can also be noticed by the means field evaluation that the highest severity was only 2.33
491 from 5 (the most severe).

492
493

494 **The improvement of multi-trait GWAS from single trait GWAS**

495 Although all single traits from a different phenotype approach had moderate to high
496 heritability, none of them provided significant peaks from GWAS from theoretically expected
497 QQ-plots that were not distributed as expected from theory. This incidence can be explained
498 by the high density of markers with high collinearity between markers and too small popula-
499 tion size, giving the low power detection (Muir, 2007). Moreover, to utilize the SNP from
500 multiple phenotype, multi-trait GWAS was suggested than aggregating the association SNP
501 from each single phenotype (Cotsapas et al., 2011). Although the low correlations among phe-
502 notyping results of BLS resistance, such a low correlation or even negative correlation can
503 lead to increase in effect size of SNP and thus increase statistical power, as happened with the
504 tetraploid group by combining DSVI and MNY (Porter & O'Reilly, 2017; Zhu et al., 2015).
505 On the other hand, combining highly positively correlated traits can reduce the average effect
506 size and power as happened in the upland group combining DTVI and DTIA. However, the
507 addition of traits did not always improve the analysis. In the lowland group, for example, only
508 mean of the two locations yielded the most significant peaks of GWAS with theoretically ex-
509 pected QQ-plot.

510 Despite the improvement of multi-trait GWAS over single-trait GWAS, 28 SNP mark-
511 ers can explain the phenotypic variance ranging from only 3.28 to 26.52% among DTVI,
512 DTIA, DSVI, DSIA, mean TL, MNY and MTL. The low PVE from GWAS is a common phe-
513 nomenon and generally caused by missing heritability and can be improved by more genomic
514 imputation and implementation of a large-scale next-generation sequence data (Fan and Song,
515 2016).

516 Moreover, each genotype group can show significant peaks from GWAS with specific
517 traits. For example, the tetraploid group only provided the significant peaks from GWAS with
518 DSVI-MNY whereas the full set of 478 genotypes provided the best fit GWAS with DTVI-
519 DTIA-DSIA. To further study resistance to BLS in switchgrass populations, single-trait
520 GWAS is not suggested due to the low power of determining significant markers (Porter &
521 O'Reilly, 2017; Zhu et al., 2015). Multi-trait GWAS from both artificial and natural inocula-
522 tion can help to improve the power.
523

524 **Dissecting resistance to BLS**

525 From the QTLs study of Sato et al. (2015), the candidate resistance genes in rice were
526 reported to be on three chromosomes with 13 SNPs markers within the LD of 200 kb (Table
527 S1). There were 278 genes linked to these markers. Among them, 172 genes were annotated
528 with 134 biological functions. There were 106 non-annotated genes. The most frequent
529 biological functions were Leucine-rich repeat family protein (6), peroxidase precursor (6),
530 transposon protein (6), nucleotide binding sites – leucine rice repeats (NBS-LRR) type disease
531 resistance protein (5), MYB family transcription factor (3), dirigent (2), pentatricopeptide
532 repeat (PPR) domain (2), fasciclin domain-containing protein (2), flavin monooxygenase (2),
533 and heavy metal associated domain (2).
534 There were only two significant SNP markers that overlap with candidate resistance
535 genes from rice Chr07N_48546809 from the 478 GWAS and Chr07K_54502620 from upland
536 GWAS (Table 1 and S7). Chr07N_48546809 linked to Pavir.7NG240300 encoding for cyto-
537 chromes P450, which involved diverse oxidation reactions and triterpene synthesis (Geisler et
538 al.,). The most well-known triterpene against fungal disease was Avenacins in oat (*Avena*

539 spp.). Chr07K_54502620 linked to Pavir.7KG238800 encoding for EGF_CA which has been
540 proved to be the main structure of *ZmWAK* conferring resistance to maize head smut by con-
541 trolling the galacturonan-binding for cell wall mediation (Zuo et al. 2015). In addition to
542 genes overlapping with the resistance rice genes, Chr07N_61547213 showed as the signifi-
543 cant peak in both 478 and 4X GWAS linked with Pavir.7NG322400 encoding for PPR repeat,
544 which is one of the potential resistance genes in rice. These PPR repeat families were com-
545 monly known for disease resistance genes (Geddy & Brown, 2007). Pavir.2NG397900 encod-
546 ing GDP-fucose protein O-fucosyltransferase relating to cell wall development and disease
547 resistance (Perrin et al., 1999).

548 Although the rest of significant peaks are unique within each group of GWAS, we
549 considered the biological functions for the resistance over the fixation of the alleles in each
550 population for better resistance mechanism. In the full set of 478, on chromosome 2N,
551 Pavir.2KG226200 was predicted to function as a zinc-binding RING finger. The zinc finger
552 domains suggested the broad-spectrum nature of rice blast resistance gene *Pi54* classed as
553 NBS-LRR, which played an important role in effector-triggered immunity (ETI) (Gupta et al.,
554 2012). Moreover, this gene also encoded glycosyltransferase, which was responsible for cell
555 wall synthesis and modification leading to disease resistance (Vorwerk et al., 2004).
556 Pavir.2NG424700 encoded for pyruvate dehydrogenase, which is a key protein in tricarbox-
557 ylic acid cycle. This can suggest the high energy intensive for resistance response as happened
558 in wheat leaf rust (*Puccinia triticina* Eriks) (Bolton et al., 2008). Pavir.2NG500000 encoded
559 for casein kinase II. The protein itself did not confer the resistance but it is often found as
560 many of motifs in Mlo (Niu & He, 2009) which was identified to provide broad resistance to

561 powdery mildew in barley (Jorgensen, 1992). On chromosome 3K, Pavir.3KG329700 en-
562 coded DNA polymerase III mainly known for DNA replications and repairing DNA damaged
563 by hydrogen peroxide (Hagensee et al., 1987). The DNA repair process is part of the plant im-
564 mune response (Song et al., 2011) especially in the infection of *B. oryzae* that hydrogen per-
565 oxide was shown to accumulate in a leaf (Kim et al., 2014). Pavir.3KG358400 encoded for
566 serine aminopeptidase conferring no resistance but playing a major role in necrosis (López-
567 Otín & Bond, 2008). On chromosome 4N, since Chr04N_47494808 was more closely linked
568 to Pavir.4NG267200 encoding for uncharacterized protein suspected of hydrolase activity, the
569 next Pavir.4NG267500 was considered for its function. The further gene encoded for Villin
570 which is a tissue-specific actin modifying protein responsible for anti-apoptotic activity sup-
571 pressing necrosis (Khurana & George, 2008; Xu et al., 2004). On chromosome 5N,
572 Pavir.5KG028600 encoded for tetratricopeptide repeat which was one of five domains of
573 SGT1 resistance gene in Arabidopsis (Azevedo et al., 2002). Pavir.5KG482000 encoded for
574 ATP-binding cassette (ABC) transporter. There are many subgroups of ABC. In Arabidopsis,
575 the mutant lacking the ABC transporter of penetration3/pleotropic drug resistance8
576 (PEN3/PDR8) conferred nonhost susceptibility to *Blumeria graminis*, suggesting that the
577 ABC transporter was involved in exporting secreted toxins from the fungus and fungal sup-
578 pressor from the host (Stein et al., 2006). Also, in wheat, the ABC suggested the exportation
579 of mycotoxin conferring resistance to Fusarium head blight (Walter et al., 2015). On chromo-
580 some 7K, Pavir.7KG196600 encoded for interleukin-1 receptor-associated kinase, which was
581 the pathogen recognition resulting in plant response (Kumagai et al., 2008). Pavir.7KG255000
582 encoded for oligo peptide transporter protein, which was also shown in rice QTL resistance to
583 rice blast (*Manaporthe grisea*) (Wang et al., 2001). Lastly for full set, on chromosome 9N,

584 Pavir.9NG844200 encoded for Mitochondrial Fe(II) transporter MMT1. Despite no direct re-
585 port of resistance from this activity, mitochondria were proven to control redox homeostasis
586 under stress (Dutilleul et al., 2003).

587 In GWAS from 4X-DSVI-MNY, on chromosome 3K, Pavir.3KG095200 encoded for
588 both peroxisomal membrane protein and ABC transporter. As the high involvement of ROS in
589 the infection of *B. oryzae* (Antonious and Jawhar, 2013), peroxidase played an important role
590 in Pathogen-associated molecular pattern-triggered immunity (PTI) (Mammarella et al.,
591 2015). Additionally, Pavir.3NG101800 on chromosome 3N encoded ABC transporter.

592 The GWAS from 8X-DTVI-DSIA yielded only one significant SNP on chromosome
593 9N. Pavir.9NG061100 encoded for DNA topoisomerase and more importantly dirigent. Diri-
594 gent protein conferred resistance by lignin and lignan synthesis (Ralph et al., 2006). The lig-
595 nin synthesis was expected to relate with resistance to BLS by strengthening cell walls
596 (Dallagnol et al., 2013).

597 In GWAS from lowland mean of two locations, on chromosome 1K, the further linked
598 Pavir.1KG225100 encoded for MORC family-ATPases. This protein is the key component of
599 compromised for recognition of Turnip Crinkle Virus (CRT1) in Arabidopsis that are required
600 for PTI, basal resistance, no-host resistance and systemic acquired resistance (Kang et al.,
601 2012). On chromosome 5N, Pavir.5NG192000 encoded for COP9 signalosome complex. In-
602 terestingly, this signalosome complex interacts with SGT1, which Pavir.5KG028600 from
603 GWAS of 478 genotype set encodes, indicating the disease resistance (Azevedo et al., 2002).
604 Pavir.5NG248900 encoded basic leucine zipper (bZIP) transcription factor, which is a key
605 modulator in hypersensitive response and salicylic acid (Alve et al., 2006). TB2/DP1,
606 Pavir.5NG476900 encoded for abcisic-acid induced TB2/DP1, HVA22 family responsible for

607 membrane turnover or decrease unnecessary secretion against Southern corn leaf blight by
608 *Cochliobolus heterostrophus* (Drechs.) Drechs. in maize (Balint-Kurti et al., 2010). On chro-
609 mosome 6N, Pavir.6NG083100 encoded homeobox-leucine zipper protein playing an im-
610 portant role in responses to abscisic acid under stress (Gago et al., 2002), transcriptional regu-
611 lation for disease resistance in rice (Luo et al., 2005) and programmed cell death (Mayda et
612 al., 1999). On chromosome 7N, Pavir.7NG091000 encoded sucrose synthase. Herbers et al.,
613 (1996) suggested that hexose sensing was important to activate defense-related genes as well
614 as repression of photosynthetic genes. On chromosome 9N, Pavir.9NG173600 encoded Phy-
615 tochrome-interacting factor 4. Photo receptors in plants plays essential role in hydrogen per-
616 oxide and ROS in the cell resulting in disease response (Karpinski et al., 2003). Lastly,
617 Pavir.9NG499500 encoded for alpha/beta hydrolase which is a diversely biochemical protein
618 family involving the activation of hydrogen peroxide (Lenfant et al., 2012).

619 In the last GWAS from upland DTIA-MTL, Chr01K_61692577 linked to two interest-
620 ing genes. First, Pavir.1KG372200 encoded Exo70 exocyst complex subunit, which was re-
621 quired for recognizing the avirulent effector AVR-Pii from rice blast resulting in ETI (Fuji-
622 saki et al., 2015). The other linked gene was Pavir.1KG372200 encoding for Nicotinamidase
623 that showed resistance to tomato mosaic virus (Arens et al., 2010).

624 Although these candidate genes from the switchgrass association studies shared some
625 functions from candidate genes from rice resistance to BLS, some of the potential functions
626 conferring resistance were still missing such as MYB. In rice, the *MYB* gene controls
627 Jasmonic acid (JA) against rice blast fungus: *Pyricularia greisea* (Ambawat et al., 2013; Lee
628 et al., 2001). In Arabidopsis interaction with necrotrophic *Alternaria brassicicola*, the re-
629 sponse to JA was the upregulated plant defensin protein (*Pdf*) gene.

CONCLUSIONS AND POTENTIAL APPLICATIONS

In conclusion, the resistance to BLS resulted differently based on different phenotyping approaches, with low correlations suggesting that resistance was sensitive to phenotyping methods in different conditions. Although it is difficult to standardize the phenotyping approach that can take account of all conditions, finer and more powerful phenotyping of the resistance is still needed. As such, to the best of our phenotyping combination, 'SW788', 'SW806', 'SW802', 'SW793', 'SW781', 'SW797', 'SW798', 'SW803', 'SW795', 'SW805 should be considered as candidates for resistance. To increase the statistical power in SNP detection, the five subgroups of populations were conducted with multi-trait GWAS. The full population of 478 genotypes showed the most SNP markers that can be considered as potential candidate genes related to resistance to BLS with reasonable biological functions. Additionally, the SNP markers from the other subgroups provided broader resistance mechanism. Nevertheless, one needs to be prudent regarding the utilization of these markers. The dependency of multitrait GWAS to yield significant SNP peaks brought the challenge for genomic-assisted breeding for resistance to BLS. The application of these markers cannot be directly transferred for genomics-assisted selection without validation to the choices of phenotyping approaches in new populations.

When considering biological functions from the GWAS regardless of subgroups and trait combinations, the defense mechanisms against BLS are complicated and multifaceted, probably controlled by multiple small-effect alleles. The defense mechanism started from basal defense via cell-wall mediated proteins and PTI via peroxidase. Then, ETI depending on NBS-LRR, and PPR repeat took place. The responses to necrosis were suppressed by homeo-

652 stasis and anti-apoptotic proteins. To our knowledge, this was first research to dissect the re-
653 sistance to BLS in switchgrass. With the current power of detection within the population,
654 multi-trait GWAS can provide functional insight into the resistance within multiple subgroups
655 of genotypes.
656

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665

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969 **TABLES**

970 Table 1. Broad-sense heritability of each trait in each groups.

971

Traits	H^2 ± s.e.				
	full set 478	**4X**	**8X**	**lowland**	**upland**
Severity from the detached leaf via vision (DTVI)	0.76 ± 0.02	0.84 ± 0.04	0.66 ± 0.06	0.87 ± 0.03	0.71 ± 0.05
Severity from the detached leaf via image analysis (DTIA)	0.74 ± 0.06	0.78 ± 0.05	0.72 ± 0.03	0.81 ± 0.06	0.68 ± 0.02
Severity from the leaf disk assay via vision (DSVI)	0.76 ± 0.05	0.70 ± 0.02	0.74 ± 0.05	0.35 ± 0.04	0.63 ± 0.04
Severity from the leaf disk assay via image analysis (DSIA)	0.5 ± 0.04	0.33 ± 0.06	0.52 ± 0.04	0.45 ± 0.05	0.14 ± 0.03
Severity in field from two locations	0.003 ± 0.06	0.003 ± 0.04	0.1 ± 0.05	0.001 ± 0.08	0.08 ± 0.06
Severity in field from NY	0.32 ± 0.05	0.31 ± 0.05	0.29 ± 0.04	0.38 ± 0.03	0.29 ± 0.04
Severity in field from PA	0.61 ± 0.04	0.68 ± 0.03	0.52 ± 0.02	0.78 ± 0.05	0.51 ± 0.03

972

973 Table 2. Markers significantly associated with resistance to Bipolaris leaf spot in each case.

Cases	SNP	Chro-mosome	p-value	Dis-tance from gene (bp)	*P. virgatum* gene	Orthologous genes	Identity	Predicted functions
478-DTVI-DTIA-DSIA	Chr02K_29538993	Chr02K	3.30E-06	1083	Pavir.2KG226200	Pahal.B01583.1[Pha]	99.6	Zinc-binding RING-finger, Glycosyl transferase family group 2, cellulose bi-osynthesis
	Chr02N_74508772	Chr02N	1.76E-05	899	Pavir.2NG397900	Pahal.B03217.1[Pha]	97.2	GDP-fucose protein O-fucosyltransferase
	Chr02N_79645558	Chr02N	1.00E-05	1496	Pavir.2NG424700	Sobic.002G259700.1[Sbi]	98.4	Pyruvate dehydro-genase (acetyl-trans-ferring)
	Chr02N_88427045	Chr02N	5.70E-06	1046	Pavir.2NG500000	Pahal.B04009.1[Pha]	100	Casein kinase II, beta subunit
	Chr03K_26598614	Chr03K	9.25E-06	1239	Pavir.3KG329700	Sevir.3G174000.1[Svi]	90.6	DNA polymerase III
	Chr03K_28867984	Chr03K	8.31E-06	788	Pavir.3KG358400	Sevir.3G185400.1[Svi]	93.3	Serine aminopepti-dase
	Chr04N_47494808	Chr04N	8.29E-06	235	Pavir.4NG267200	Sobic.010G215800.1[Sbi]	78.2	similar to OSIGBa0118P15.3 relating to protein hydrolase activity
				8698	Pavir.4NG267500	LOC_Os06g44890.1[Osa]	93.3	Villin as an anti-apoptotic protein
	Chr05K_5450256	Chr05K	7.26E-06	47	Pavir.5KG028600	Pahal.E04447.1[Pha]	97.5	Tetratricopeptide re-peat
	Chr05K_90814719	Chr05K	8.62E-07	321	Pavir.5KG482000	Zm00008a014067_T0[Zma]	94.9	ABC transporter
	Chr07K_50656672	Chr07K	1.22E-05	207	Pavir.7KG196600	Zm00008a007144_T01[Zma]	95.2	interleukin-1 recep-tor-associated kinase

Cases	SNP	Chro-mosome	p-value	Dis-tance from gene (bp)	*P. virgatum* gene	Orthologous genes	Identity	Predicted functions
	Chr07K_56339702	Chr07K	4.94E-06	756	Pavir.7KG255200	Pahal.G01334.1[Pha]	92.1	Uncharacterized ACR, COG1399
				930	Pavir.7KG255000	LOC_Os04g44320.1[Osa]	84.2	Oligopeptide trans-porter protein
	Chr07N_48546809	Chr07N	9.13E-07	524	Pavir.7NG240300*	LOC_Os04g39430.1[Osa]	88.1	Cytochrome P450 CYP4/CYP19/CYP2 6 subfamilies
	Chr07N_61547213	Chr07N	2.18E-07	571	Pavir.7NG322400	LOC_Os04g51350.1[Osa]	91.1	PPR repeat family
	Chr09N_106831822	Chr09N	5.42E-06	20	Pavir.9NG844100	Sobic.001G445100.1[Sbi]	73.5	Putative uncharacter-ized protein
				9902	Pavir.9NG844200	GRMZM2G118497_T0[Zma]	98.5	Mitochondrial Fe2+ transporter MMT1 and related transport-ers, metal tolerate
4X-DSVI-MNY	Chr03K_8647071	Chr03K	3.12E-06	733	Pavir.3KG095300	Sobic.009G005500.1[Sbi]	93.6	Uncharacterized po-tentially histone acetyltransferases subunit 3
				6957	Pavir.3KG095200	Sobic.009G005400.1[Sbi]	96.8	Similar to peroxiso-mal membrane pro-tein ABC transporter homologue
	Chr03N_12119852	Chr03N	7.69E-07	2038	Pavir.3NG101800	LOC_Os12g22284.1[Osa]	89.3	ABC-2 type trans-porter
	Chr07N_61547213	Chr07N	1.66E-06	571	Pavir.7NG322400	LOC_Os04g51350.1[Osa]	91.1	PPR repeat family

Cases	SNP	Chro-mosome	p-value	Dis-tance from gene (bp)	*P. virgatum* gene	Orthologous genes	Identity	Predicted functions
8X-DTVI-DGA	Chr09N_3891068	Chr09N	2.66E-07	535	Pavir.9NG061100	Sobic.001G040600.1[Sbi]	94.3	DNA topoisomerase (ATP-hydrolyzing), dirigent
low-mean TL	Chr01K_36400224	Chr01K	3.08E-06	1354	Pavir.1KG225400	Sobic.004G141800.2[Sbi]	96	Similar to Os02g0469200 protein
				10167	Pavir.1KG225100	Sevir.1G159400.1[Svi]	88	MORC family ATPases
	Chr05N_24540124	Chr05N	5.43E-06	1020	Pavir.5NG192000	Sevir.5G038000.1[Svi]	100	COP9 signalosome complex
	Chr05N_35504012	Chr05N	2.47E-06	360	Pavir.5NG248900	Sevir.5G193800.1[Svi]	88.5	basic leucine zipper (bZIP) transcription factor
	Chr05N_84742282	Chr05N	5.20E-06	1309	Pavir.5NG476900	Zm00008a032406_T01[Zma]	71.5	TB2/DP1, HVA22 family
	Chr06N_14870066	Chr06N	2.40E-06	1766	Pavir.6NG083100	Sevir.6G037200.1[Svi]	97.6	homeobox-leucine zipper protein
	Chr07N_22055455	Chr07N	1.89E-05	1088	Pavir.7NG091000	Sobic.010G276700.1[Sbi]	98.2	Sucrose synthase
	Chr09N_17627471	Chr09N	1.47E-05	2258	Pavir.9NG173600	Pahal.I02469.1[Pha]	92.7	Phytochrome-interacting factor 4
	Chr09N_82637734	Chr09N	1.16E-06	1030	Pavir.9NG499500	Zm00008a001876_T01[Zma]	95.3	Alpha/beta hydrolase family
up-DGA	Chr01K_61692577	Chr01K	1.52E-06	6831	Pavir.1KG372200	Sobic.010G272700.1[Sbi]	53.6	Exo70 exocyst complex subunit
				2646	Pavir.1KG372200	Zm00008a022268_T01	95.9	Nicotinamidase

Cases	SNP	Chro-mosome	p-value	Dis-tance from gene (bp)	*P. virgatum* gene	Orthologous genes	Identity	Predicted functions
	Chr07K_54502620	Chr07K	4.48E-06	3810	Pavir.7KG238800*	LOC_Os04g43730.1[Osa]	73.6	Calcium-binding EGF domain (EGF_CA), Wall-associated receptor kinase galacturonan-binding

974 * *P. virgatum* genes that overlapping with resistance genes in *Oryza sativa* search

975 [Osa] gene from *Oryza sativa*

976 [Pha] gene from Panicum hallii

977 [Sbi] gene from Sorghum bicolor

978 [Svi] gene from Setaria viridis

979 [Zma] gene from Zea may

980

981

982 Table 3 Phenotypic variance explained (PVE) (adjusted PVE) by accumulative 27 significant

983 SNP markers across all analyses for each trait.

Traits	PVE (adjusted PVE)
DTVI	11.68 (6.38)
DTIA	8.76 (3.28)
DSVI	25.03 (20.53)
DSIA	30.68 (26.52)
mean TL	15.73 (10.67)
MNY	15.74 (10.68)
MTL	13.7 (8.52)

984

985 Abbreviation: BLUPs of severity from the detached leaf via vision (DTVI), BLUPs of sever-

986 ity from the detached leaf via image analysis (DTIA), BLUPs of severity from the leaf disk

987 assay via vision (DSVI), BLUPs of severity from the leaf disk assay via image analysis

988 (DSIA), mean of field evaluation of BLS in two locations (mean TL), highest score of BLS in

989 NY (MNY), and highest score of BLS in two locations (MTL).

FIGURES

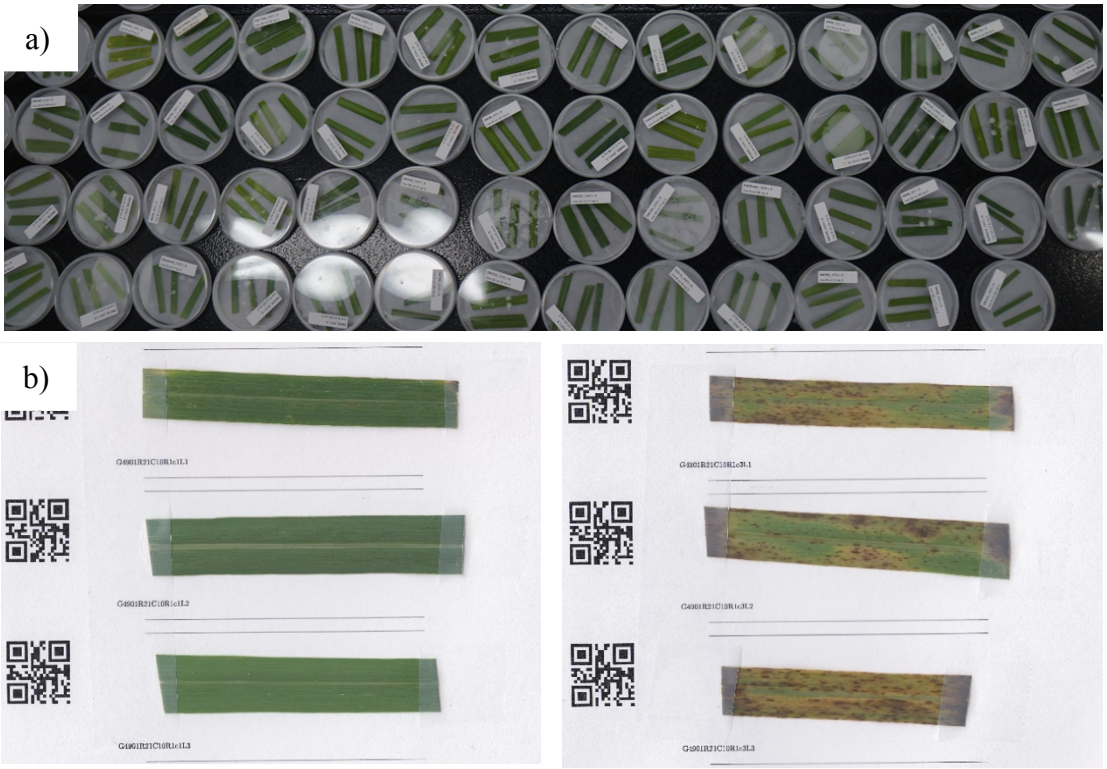

Figure 1. Detached leaf assay; a) sealed plates of detached leaves under 12-h light 25°C; b) control inoculated (left) and inoculated leaves (right) at 7 dpi were taped on white paper with QR code label for scanning for image analysis.

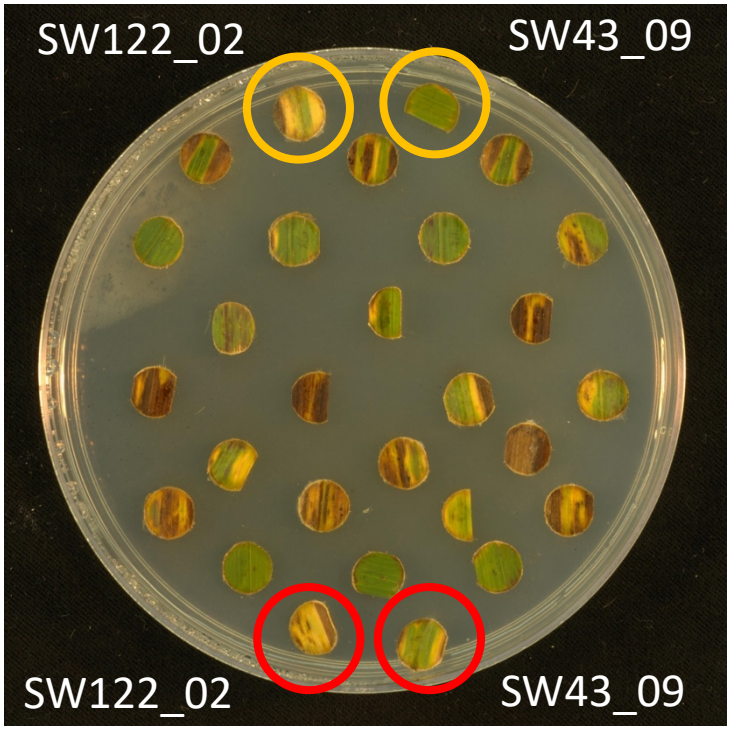

Figure 2. A plate example of leaf disk assay at 7 dpi. Each plate consisted of 28 leaf disks. SW43_09 was used as a resistant check and SW122_02 as a susceptible check. Two non-inoculated control leaf disks (yellow circles) and two inoculated leaf disks (red circles) were included in all plates. The rest of the leaf disks (24) consisted of eight genotypes with three replicates placed in a consecutive row from left to right.

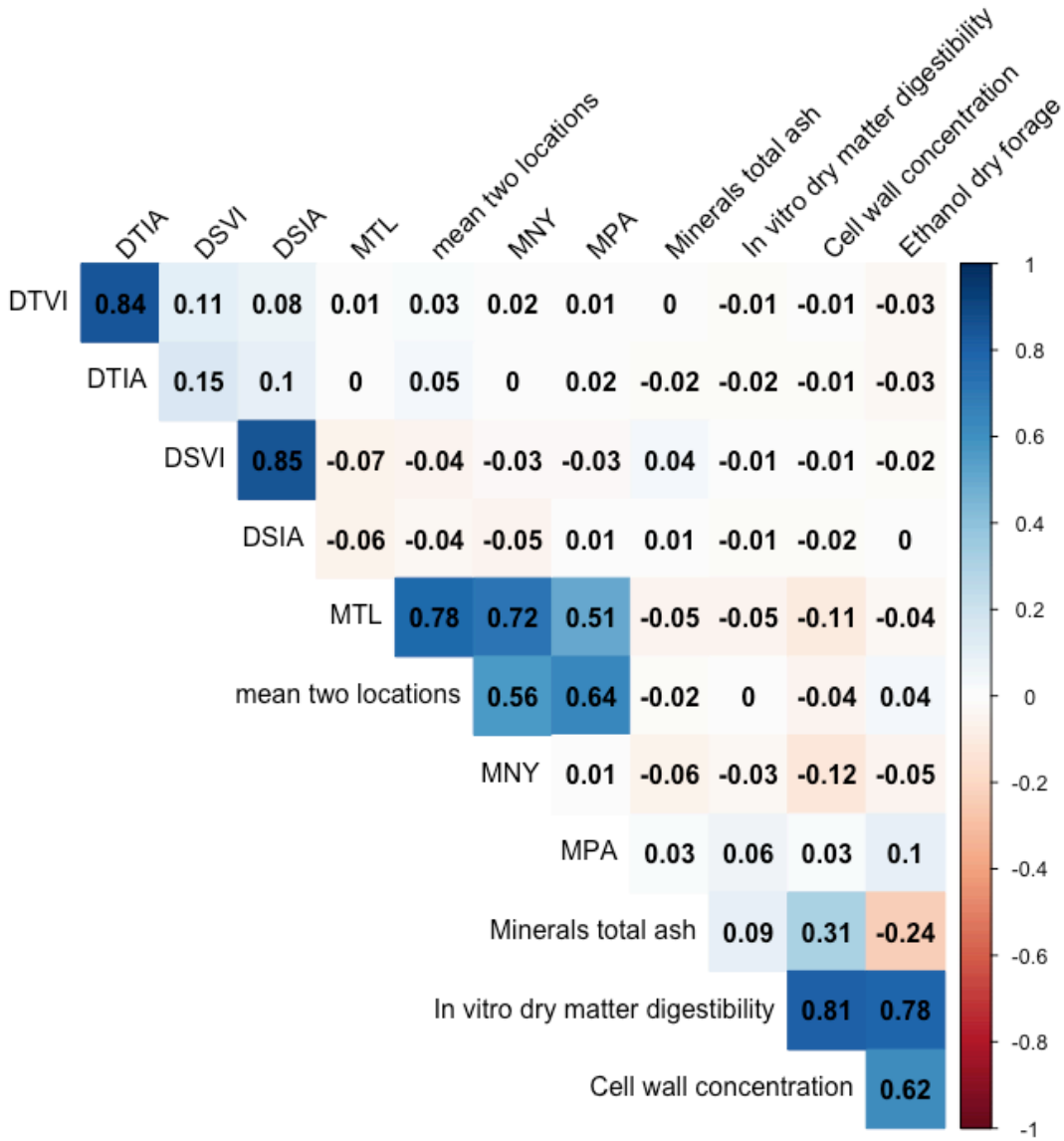

Figure 3. Correlation plot among BLUPs of severity from the detached leaf via vison (DTVI), via image analysis (DTIA), from leaf disk assay via vision (DSVI), via image analysis (DSIA), the highest score between two locations (MTL), mean from two location (TL), minerals total ash, *in vitro* dry matter digestibility, cell wall concentration, and ethanol conversion per day forage.

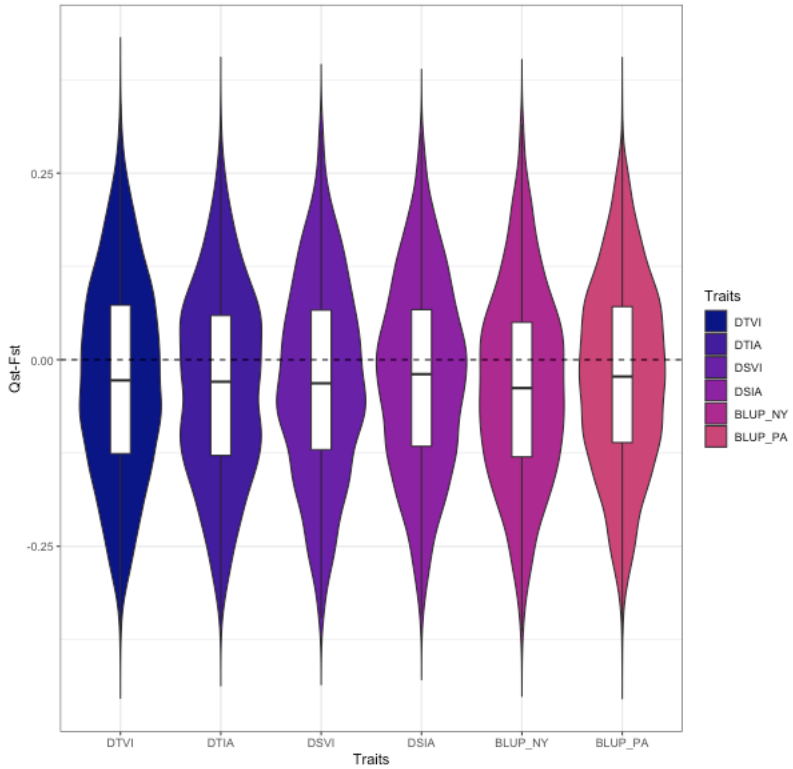

Figure 4. The bootstrapped (1000 times) distributions of Qst-Fst for each phenotype (DTVI, DTIA, DSVI, DSIA, BLUPs NY and BLUP PA) are compared to the expected value of zero under neutrality (black dashed line).

1003

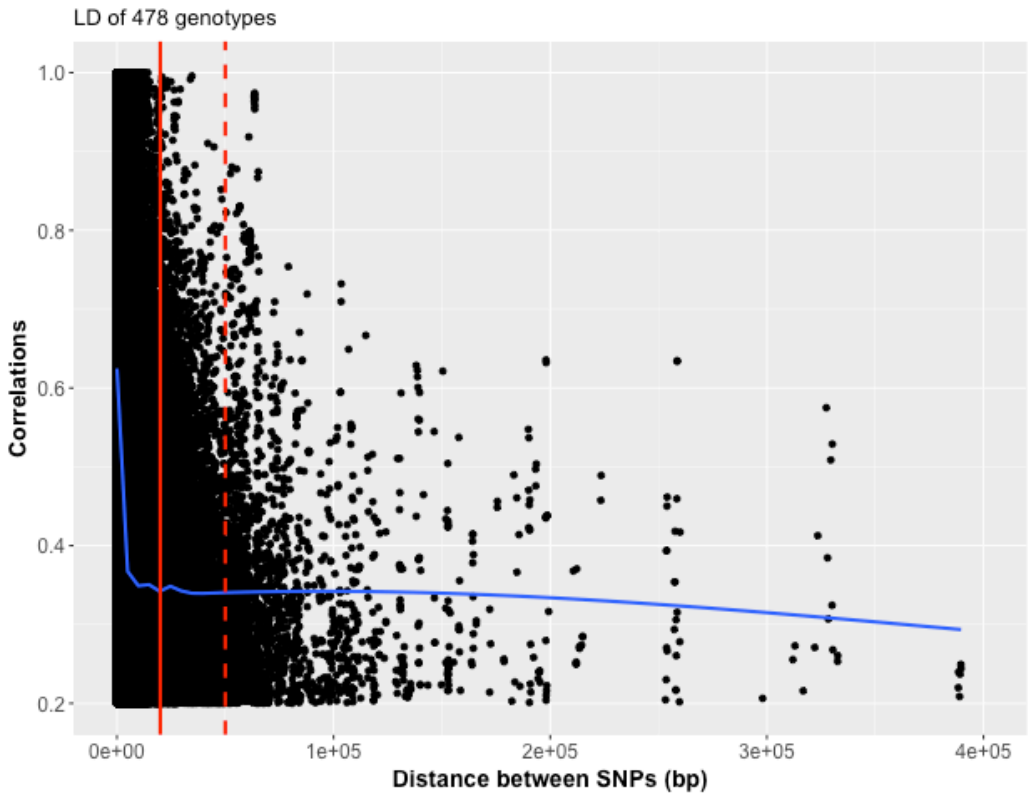

Figure 5. Scatter plot showing the linkage disequilibrium (LD) decay by plotting physical distance in base pairs against the LD estimate as correlations (R^2) in 478 genotypes. The LD (blue curve) decays rapidly within 20 kb (red line) and reaches background levels around 50 kb (dashed red line).

1004

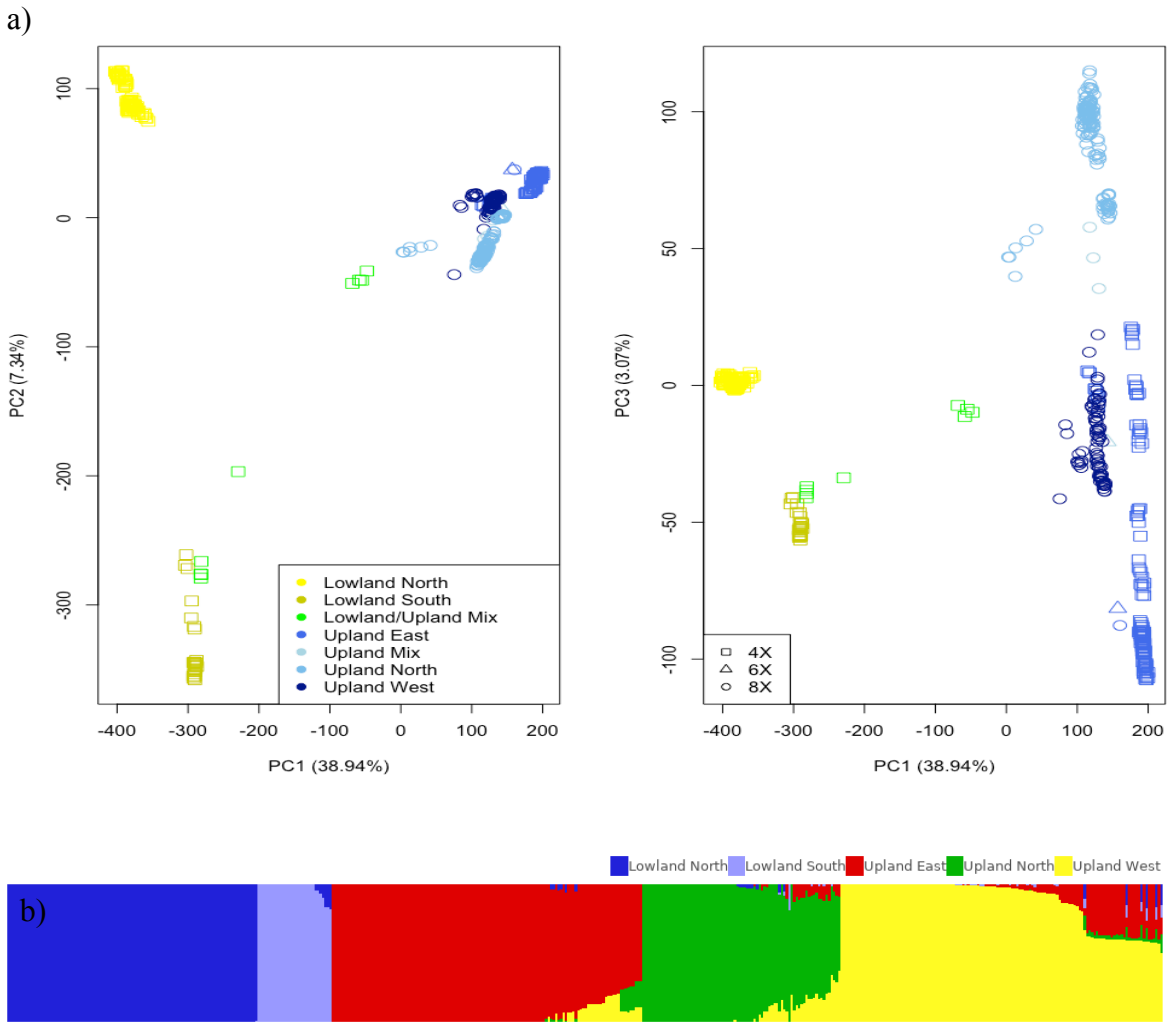

Figure 6. a) The 478 switchgrass genotypes from the Northern Association Panel showing the different distributions based on ecotype and region from principal component analysis (PC). Colors represented different ecotypes and shapes represented different ploidy levels. Percentage variances explained by PCs are in parentheses. The lowland ecotype can be differentiated into two regions from PC1 and PC2, and the upland ecotype can be grouped into three regions from PC1 and PC3. b) Admixture also showed the separations of the genotypes into five gene pools.

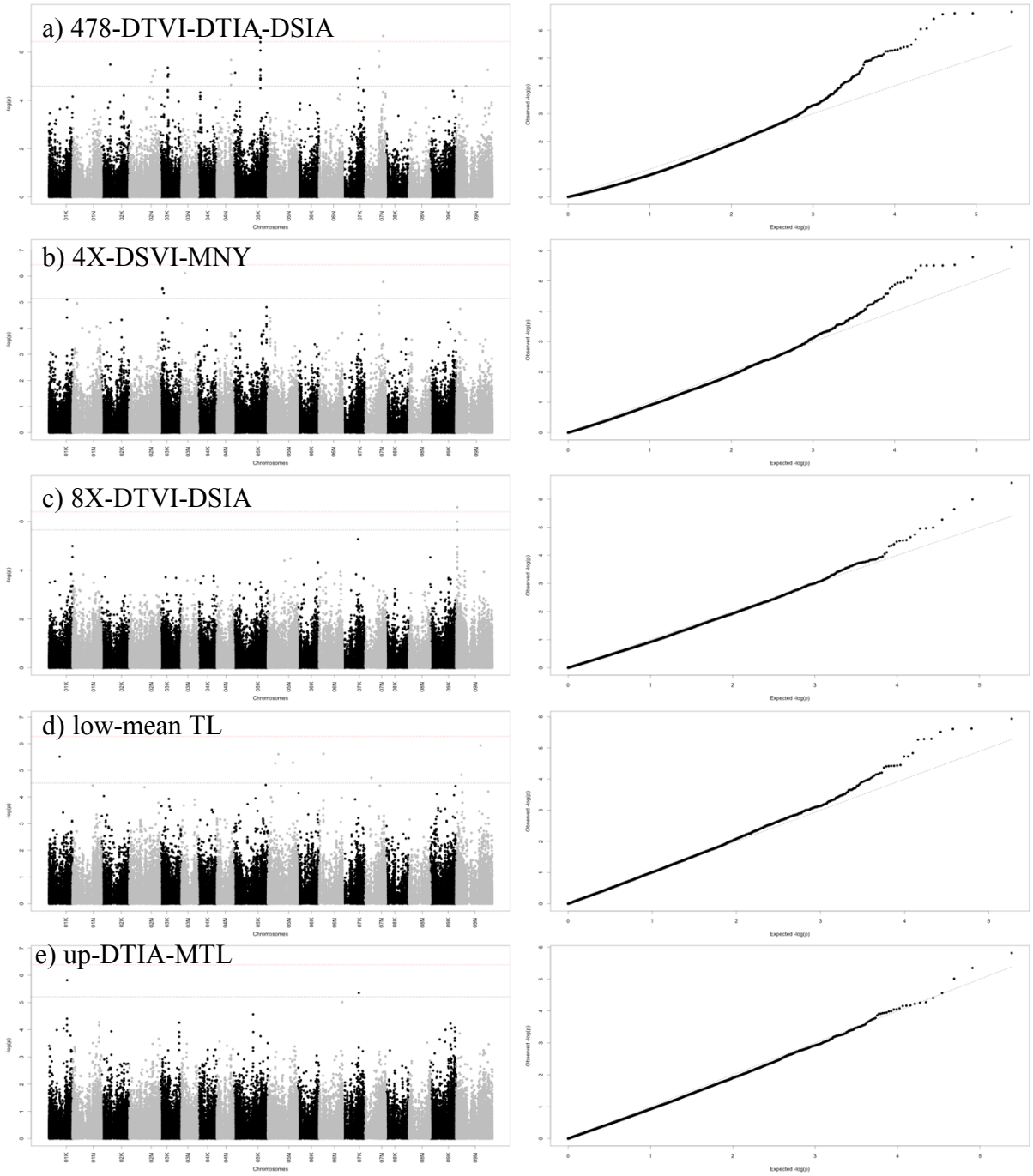

Figure 7. (Left) Manhattan plot showing genetic associated of a) DTVI-DTIA-DSIA in 478 genotypes, b) DSVI-MNY in 4X genotypes, c) DTVI-DSIA in 8X genotypes, d) mean TL in lowland ecotype, e) DTIA-MTL in upland genotypes. The black dashed line represents the FDR threshold (0.1) and the red dashed line represents Bonferroni correction threshold. On x-axis, the physical positions of the SNPs were aligned in 18 chromosomes of *P. virgatum*. (Right) Quantile-quantile (QQ) plots between the distributions of observed to expected P-values for GWAS of each trait combination in each genotype group.

Abbreviation: Abbreviation: BLUPs of severity from the detached leaf via vision (DTVI), BLUPs of severity from the detached leaf via image analysis (DTIA), BLUPs of severity from the leaf disk assay via vision (DSVI), BLUPs of severity from the leaf disk assay via image analysis (DSIA), mean of field evaluation of BLS in two locations (mean TL), highest score of BLS in NY (MNY), and highest score of BLS in two locations (MTL).

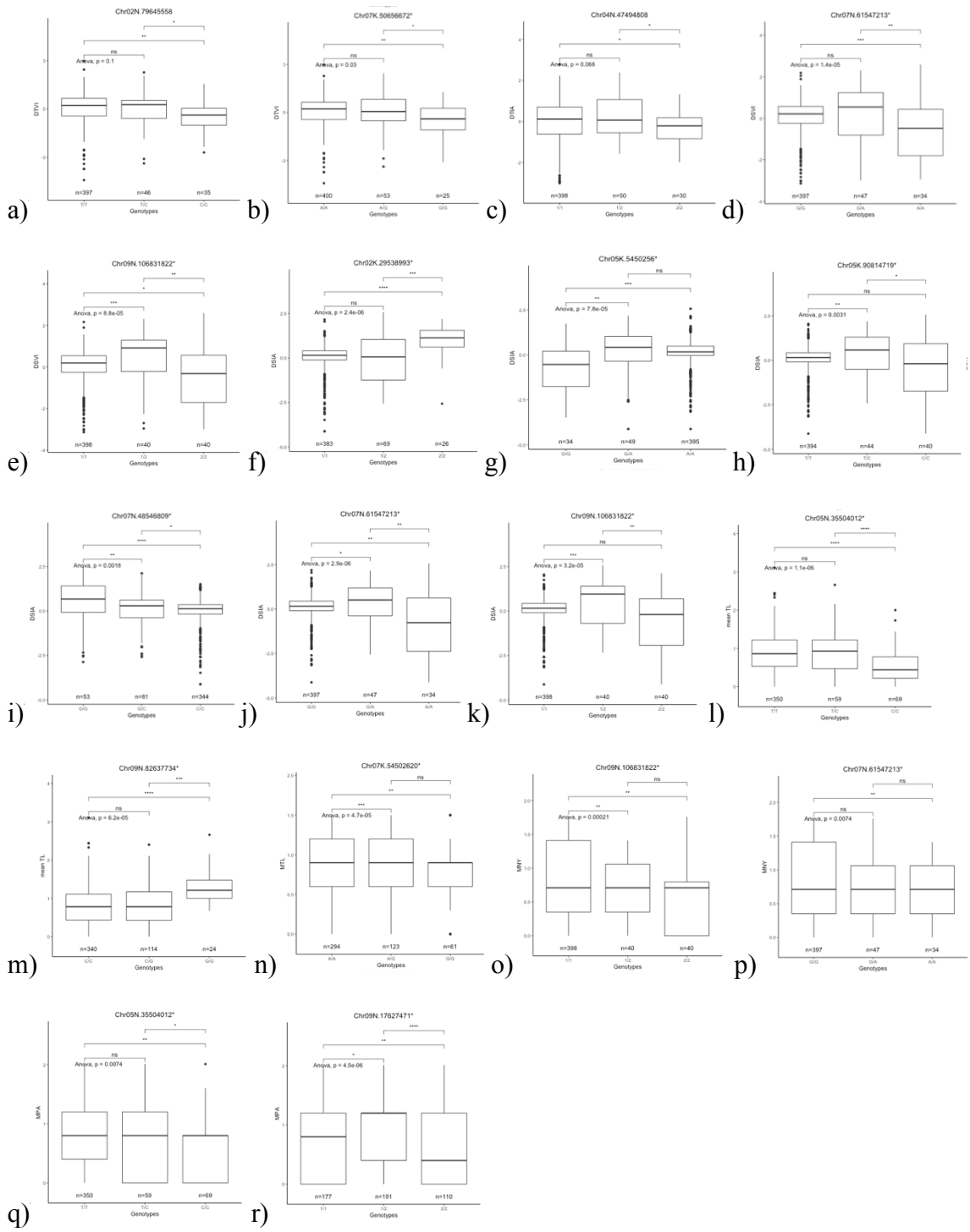

Figure 8. Examples of changes in DTVI, DTIA, DSVI, DSIA, mean TL, MTL, MNY and MPA associated with alleles at selected candidate single nucleotide polymorphisms (SNPs) in 478 genotypes. The effects of three different allele combinations were tested at alpha = 0.05. “ns” means non-significant, “*” means significant at alpha 0.05, “**” means significant at alpha 0.01, and “***” means significant at alpha 0.001.