Breeding Science 71: 365–374 (2021)
doi: 10.1270/jsbbs.20137

Research Paper

Detection of a major QTL related to smut disease resistance inherited from a Japanese wild sugarcane using GRAS-Di technology

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Smut disease of sugarcane causes considerable yield losses and the use of resistant varieties is the best control practice. Our group identified a Japanese wild sugarcane with highly smut disease resistance named 'Iriomote8'. In this study, we conducted QTL analysis for smut disease resistance using a mapping population derived from a resistant variety 'Yaenoushie', in which resistance is inherited from 'Iriomote8'. We identified 4813 non-redundant markers using GRAS-Di technology and developed a linkage map of mapping parents. We evaluated smut disease resistance of the mapping population by the inoculation test. Consequently, a large number of clones did not show the disease symptoms and the distribution of smut disease incidence tended to be "L shaped". Composite interval mapping detected an identical QTL for indices of smut disease incidence with a markedly high LOD score (26.6~45.6) at the end of linkage group 8 of 'Yaenoushie'. This QTL explained approximately 50% of the cases of smut disease incidence. In the mapping population, there were no correlations between the indices of smut disease incidence and other agronomic traits. In conclusion, this QTL could be used for marker-assisted selection to significantly improve smut disease resistance without negative effects on other agronomic traits.

Key Words: smut disease resistance, *Saccharum spontaneum*, QTL analysis, GRAS-Di technology.

Introduction

Sugarcane (*Saccharum* spp.) is the 19th largest crop in the world based on harvested area, and eighty percent of sugar consumed in the world is produced from sugarcane (FAO 2020). Sugarcane is used in the production of sugar as well as various products such as bioethanol and rum from cane juice, and paper from bagasse. In Japan, sugarcane is mainly cultivated in the south west islands of Kagoshima and Okinawa Prefectures, and it is a major crop in this region. Smut disease of sugarcane is caused by the fungus *Sporisorium scitamineum* (previously called *Ustilago scitaminea*) and found in most sugarcane producing region in the world. Smut disease is mainly characterized by the development of a whip-like structure (**Supplemental Fig. 1**), which causes considerable yield loss and reduction in cane quality. Estimates of economic losses from smut disease ranged from negligible to 75% (Carvalho *et al.* 2016, Sundar *et al.* 2012). In Japan, smut disease has been found in agricultural fields since 1930. From the 1930s to 1970s, smut disease was not observed as a result of the spread of resistant varieties, 'POJ2725' and 'POJ2878'. However, smut disease recurred in 'NCo310' at Ishigaki Island in 1972 (Yamauchi 1973) and was found throughout Okinawa Prefecture in 2001 because of the spread of the susceptible variety 'Ni9' (Teramura *et al.* 2018). 'NCo310'

Communicated by Donghe Xu
Received October 23, 2020. Accepted February 23, 2021.
First Published Online in J-STAGE on June 19, 2021.
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and 'Ni9' show high yields in ratoon crop. There are no effective fungicides that affect after smut infection and the use of resistant varieties is the best control practice. For this reason, it is necessary to develop a variety that has both smut disease resistance and high productivity. In the sugarcane breeding program of Kyushu Okinawa Agricultural Research Center NARO (KARC/NARO), smut disease resistance is evaluated by the artificial inoculation test (the wounding method). Due to limitation in the size of the test plots and the need to prepare many buds, only about 100 clones per year can be evaluated at later selection stages. An effective method of evaluating smut disease resistance at earlier selection stages, such as marker-assisted selection (MAS), is necessary to accelerate the breeding of smut disease resistant varieties.

Reliable markers that are tightly linked to genes or genomic regions controlling target traits are necessary for MAS. However, understanding the quantitative traits and mapping their loci in the sugarcane genome are challenging because of the highly polyploid and heterozygous characteristics of the genome ($2n = 100$ to 130). Modern sugarcane varieties derive from several interspecific crosses performed a century ago with the aim of incorporating the disease resistance, hardiness and tillering ability of wild species (*Saccharum spontaneum*, $2n = 5x = 40$ to $16x = 128$) into the sweet and thick noble cane (*Saccharum officinarum*, $2n = 8x = 80$) (Berding and Roach 1987, D'Hont *et al.* 1996). These interspecific crosses produced the heterozygous characteristic of sugarcane genome. There are previous reports on the detection of major quantitative trait loci (QTLs) related to disease resistance and the development of molecular markers for sugarcane breeding. Brown rust disease resistance gene, *Bru1* is the first major gene identified in sugarcane (Daugrois *et al.* 1996). Several molecular markers linked to *Bru1* were developed using the genetic map of the donor variety 'R570' (Asnaghi *et al.* 2004, Costet *et al.* 2012a). Yellow spot disease resistance (Aljanabi *et al.* 2007), yellow leaf disease resistance (Costet *et al.* 2012b) and orange rust disease resistance (Yang *et al.* 2018) are the other traits of which major QTLs were detected in sugarcane.

Smut disease resistance is thought to involve internal and external disease resistance mechanisms controlled by several small-effect QTLs. External resistance is evaluated by the dipping method in which buds are immersed briefly in a suspension of teliospores. Smut infection into the meristem in the bud occurs between 6 and 36 hours after teliospore deposition on buds (Alexander and Ramakrishnan 1980). In the case of external resistance mechanisms, hyphae of *S. scitamineum* cannot penetrate through the bud scale because host plants modify the cell wall structure and component, such as the accumulation of lignin and phenolic compounds in epidermis (Marques *et al.* 2018). Internal resistance is expressed after *S. scitamineum* penetrates through the bud scale and is governed by several defense responses induced in sugarcane. Internal resistance is evaluated by the wounding method (Burner *et al.* 1993, McNeil *et al.* 2018). The dipping method is not effective for the detection of marker-trait associations because disease incidence is very low (Raboin *et al.* 2003). Therefore, we focused on internal resistance evaluated by the wounding method.

Many studies have been carried out to elucidate the internal resistance mechanism. Lloyd and Pillay (1980) found that the rate of colonization by *S. scitamineum* in sugarcane tissue correlated with the resistance ratings using microscopy. Carvalho *et al.* (2016) observed that *S. scitamineum* showed intercellular growth in a resistant variety (SP80-3280) and intense intracellular growth in a susceptible clone (IAC66-6) using GFP-tagged strains. To evaluate plant defense responses, Que *et al.* (2014) compared gene expression following wounding inoculation between 'Yacheng05-179' (resistant variety) and 'ROC22' (susceptible variety), and clarified that the largest difference occurred at 48 hours after inoculation compared with 24 and 120 hours after inoculation. Differentially expressed genes were involved in plant hormone signal transduction, flavonoid biosynthesis, plant-pathogen interaction, cell wall fortification pathway and other resistance-associated metabolic pathways. The accumulation of reactive oxygen species (ROS) in plant tissue is known to contribute to pathogen growth inhibition. Peters *et al.* (2017) observed that a resistant variety (SP80-3280) accumulated ROS in buds at 72 hours after inoculation. Because the sugarcane response to smut infection is diverse and the sugarcane genome is complex, no major QTLs associated with smut disease resistance have been detected.

KARC/NARO conserves approximately 200 accessions of Japanese *Saccharum spontaneum* collected by NARO Genebank project. Our group identified highly smut disease resistant accessions within this collection (Sakaigaichi *et al.* 2018) and developed mapping populations using the identified resistant accessions. Toyota Motor Corporation invented Genotyping Random Amplicon Sequence-Direct (GRAS-Di) technology, which enables rapid and reproducible genotyping of sugarcane (Enoki 2019, Enoki and Takeuchi 2018). In this study, we conducted QTL analysis for smut disease resistance using GRAS-Di technology and the mapping population derived from a resistant variety 'Yaenoushie', in which resistance is inherited from the resistant accession 'Iriomote8'.

Materials and Methods

Plant materials

The mapping population consisted of 154 clones derived from a 'Yaenoushie' × 'KY08-129' cross (**Fig. 1**). 'Yaenoushie' is a highly smut disease resistant variety for forage use derived from a 'NiF8' × 'Iriomote8' cross (Sakaigaichi *et al.* 2019). 'Iriomote8' (175508, Genebank Project, NARO) is a *S. spontaneum* accession collected from Iriomote Island, Okinawa, Japan and acted as the

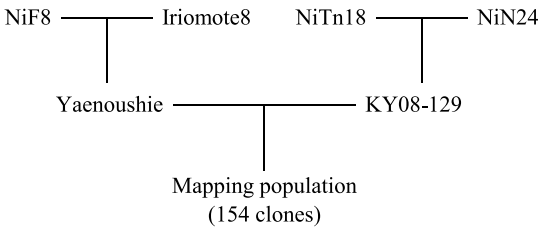

Fig. 1. The pedigree record of the mapping population.

donor of the smut disease resistance of 'Yaenoushie'. 'KY08-129' is a clone derived from a 'NiTn18' × 'NiN24' cross. 'NiF8', 'NiTn18' and 'NiN24' are Japanese sugarcane varieties developed by KARC/NARO. In the preliminary study, smut disease resistance of 'Yaenoushie' and 'KY08-129' was evaluated as highly resistant and moderately resistant, respectively. We extracted DNA from the mapping population and mapping parents using DNeasy Plant Mini Kit (QIAGEN, Germany) and obtained genotype data using GRAS-Di technology (Toyota Motor Corporation, Japan). In the first step of GRAS-Di technology, arbitrary fragments of each sample were amplified by random primers. Details of the PCR procedure are described in Hosoya *et al.* (2019). PCR products were sequenced as 100 bp paired-end reads using a next generation sequencer (HiSeq 4000, Illumina, USA). We constructed two linkage maps for each mapping parent using single-dose markers, as the number of sugarcane chromosome is not constant and there is no reference genome for sugarcane. Single-dose markers are known to be effective in the genetic map construction of polyploid species (Yang *et al.* 2017). For example, if dominant single-dose markers are present in one parent, these markers segregate at a 1:1 ratio in the progeny. We detected genetic polymorphisms found only in one place in the genome and in only one parent using GRAS-Di software (Toyota Motor Corporation, Japan) and generated 29,380 dominant single-dose markers. We eliminated markers with missing data and integrated multiple markers showing the same genotype data into one marker. Ultimately, we identified 4813 non-redundant markers. We constructed the linkage map of 'Yaenoushie' using 2936 markers and 'KY08-129' using 1877 markers with the linkage mapping program AntMap ver. 1.2 (Iwata and Ninomiya 2006). Genetic distances between markers were computed using the Kosambi mapping function with the program.

Evaluation of smut disease resistance

Smut disease resistance of the mapping population was evaluated by the smut inoculation test in 2018 and 2019. Teliospores were collected by Okinawa Prefectural Agricultural Research Center (Itoman, Okinawa, Japan) in the year before the experiments. They were homogenized before storage and checked for viability before inoculation. The race of smut pathogen thought to be identical throughout the south west islands of Japan, based on the reactions of several indicating varieties (Yamauchi 1989). Smut inoculation was conducted using the pin-prick wounding method described in Shih *et al.* (1981). We prepared 40 buds per clone in 2018, 20 buds per clone in 2019, and pricked 6 holes at the base of each bud using injection needles (NN-2538R, Terumo, Japan). Subsequently, a 10% w w^{-1} suspension of teliospores was applied around the pricked points using brush. After inoculation, the buds were kept in the dark for three days. The air temperature and humidity were controlled at approximately 30°C and 100%RH. We planted the inoculated buds in cell trays (#50, Tokan Kosan, Japan) filled with culture soil and grew them in a greenhouse from April to November 2018 and from May to November 2019 (approximately 200 days) (**Supplemental Fig. 1**). The condition in the greenhouse during the test was maintained at high temperature and high humidity suitable for smut development. We counted the number of whip-developed plants every two weeks and calculated the percentage of whip emergence by the following formula.

Percentage of whip emergence (%) = Number of whip-developed plants/Number of germinated plants × 100

In this study, we used another index of smut disease incidence, the percentage of infected plants, calculated by the following formula.

Percentage of infected plants (%) = Number of infected plants/Number of germinated plants × 100

We detected the smut infection of each plant by PCR using primers, UscITS1 (5′-AGGTGTGGCTCGCACCTGTCTA-3′)/UscITS2 (5′-ATCCTCACCACCAAAGTCCTGA-3′) specific to *S. scitamineum* (Sugisawa *et al.* 2002). We sampled leaves from the middle part of each plant at the inoculation test plot from 10 to 13 July 2018 (approximately 80 days after inoculation). We did not sample in 2019 because the growth of the plants was delayed due to a delay in inoculation date. We extracted crude lysate for each sample using Lysis Buffer for PCR (Takara Bio, Japan), which were subsequently used as templates for PCR. The reaction mixture consisted of 12.5 µL of Gflex PCR buffer (Takara Bio, Japan), 0.5 µL of Gflex DNA Polymerase (Takara Bio, Japan), 0.5 µL of each primer, 9.0 µL of sterile distilled water, and 2.0 µL of lysate of each sample. The total volume of the reaction mixture was 25 µL. The PCR conditions were as follows. The initial denaturation was 94°C for 1 min. Thermal cycling consisted of 40 cycles of denaturation at 94°C for 15 sec, annealing and extension at 68°C for 1 min. The final extension was 68°C for 4 min. We detected PCR products using a microchip electrophoretic machine (MCE-202 MultiNA, Shimadzu, Japan).

QTL analysis for smut disease resistance

We conducted composite interval mapping using the 'qtl'

package in R 3.5.1. We distributed pseudo markers at 1 cM intervals. We set the number of marker covariations and a window size at 12 and 10 cM, respectively. To obtain the logarithm of the odds (LOD) score threshold ($P < 0.05$), 1000 permutations were run by randomly shuffling the trait values.

Evaluation of agronomic traits

To evaluate agronomic traits of the mapping population, we cultivated them in the experimental field of Tanegashima Sugarcane Breeding Site of KARC/NARO (30°43′N, 131°04′E, altitude: 45 m) from February 2017 to January 2019. The individual plot size was 1.65 m^2 (1.5 m long × 1.1 m width of row) and plant density was twelve plants per plot (7.27 plants m^{-2}). We planted one-bud stem cuttings and applied chemical fertilizer as a basal dressing (7.2 g N m^{-2}, 12.0 g P_2O_5 m^{-2}, 6.0 g K_2O m^{-2}) on 24 February 2017. Subsequently, we applied chemical fertilizer as top dressings for plant canes (4.5 g N m^{-2}, 4.5 g K_2O m^{-2}) on 18 May and 14 June 2017. In order to evaluate ratoon canes, we removed all parts of the plant above ground by a cane harvester and applied chemical fertilizer as a basal dressing (7.2 g N m^{-2}, 12.0 g P_2O_5 m^{-2}, 6.0 g K_2O m^{-2}) on 12 April 2018. Subsequently, we applied chemical fertilizer as a top dressing for ratoon canes (9.0 g N m^{-2}, 9.0 g K_2O m^{-2}) on 13 June 2018. We counted the number of stalks in each plot in January 2018 and October 2018. We measured the stalk height in July 2017, and the Brix value in January 2018 and January 2019 using a hand refractometer (Master, Atago, Japan). We performed Shapiro-Wilk test and correlation analysis for phenotype data using R 3.5.1.

Results

Phenotypic evaluation

The frequency distributions of measured traits in the mapping population are shown in **Fig. 2**. We performed the Shapiro-Wilk test to confirm whether the distributions were normal. The frequency distributions of smut disease incidence were distinct from the normal distribution at a 0.1% level, and tended to be "L shaped" with a large number of resistant clones on the left tail and a small number of susceptible clones spread along the right tail. The frequency distributions of number of stalks of plant canes and brix value of plant canes were distinct from the normal distribution at a 5% level. The frequency distributions of temporary

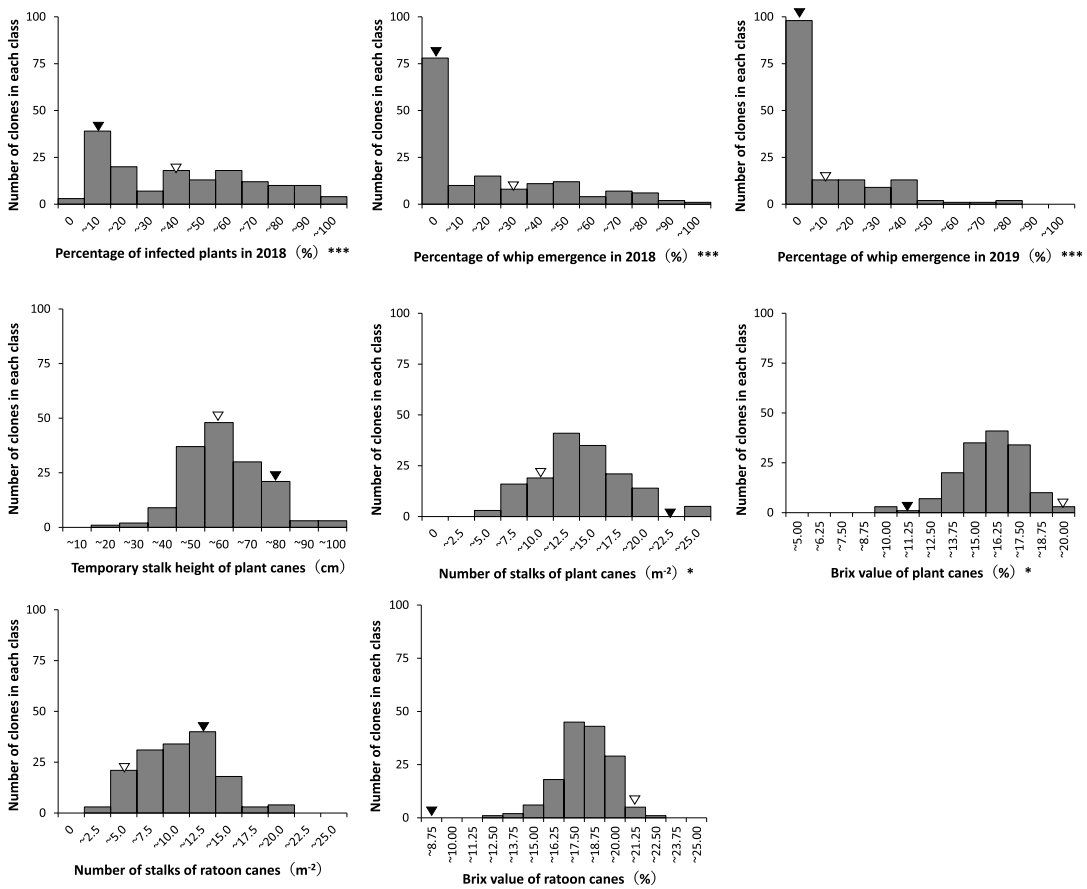

Fig. 2. The frequency distributions of measured traits in the mapping population. Black triangles (▼) indicate positions of 'Yaenoushie'. White triangles (▽) indicate positions of 'KY08-129'. * and *** indicate that the distribution is significantly distinct from the normal distribution at 5% and 0.1% level, respectively.

Table 1. Correlation coefficients between measured traits of the mapping population

Measured traits	N	Percentage of infected plants in 2018	Percentage of whip emergence in 2018	Percentage of whip emergence in 2019	Temporary stalk height of plant canes	Number of stalks of plant canes	Brix value of plant canes	Number of stalks of ratoon canes
Percentage of infected plants in 2018	154	–	–	–	–	–	–	–
Percentage of whip emergence in 2018	154	0.815[a] ***[b]	–	–	–	–	–	–
Percentage of whip emergence in 2019	152	0.665***	0.786***	–	–	–	–	–
Temporary stalk height of plant canes	154	–0.083	–0.110	–0.013	–	–	–	–
Number of stalks of plant canes	154	0.086	0.068	0.015	0.087	–	–	–
Brix value of plant canes	154	0.052	0.000	0.049	–0.053	0.008	–	–
Number of stalks of ratoon canes	154	0.011	0.088	0.079	–0.118	0.469***	0.072	–
Brix value of ratoon canes	150	0.039	0.038	0.051	0.045	0.071	0.271***	–0.048

[a] The underlined values indicate Spearman's rank correlation coefficients. The other values indicate Pearson's product-moment correlation coefficients.
[b] *** indicates a significant correlation at a 0.1 % level.

stalk height of plant canes, number of stalks of ratoon canes and brix value of ratoon canes each followed normal distributions.

Subsequently, we evaluated the relationship between measured traits of the mapping population (**Table 1**). There were significant correlations among the indices of smut disease incidence ($P < 0.001$). On the other hand, there were no significant correlations between indices of smut disease incidence and other agronomic traits.

Construction of linkage maps and QTL analysis for smut disease resistance

We constructed a linkage map of 'Yaenoushie' using 2936 markers. These markers coalesced into 117 linkage groups (**Table 2**). The cumulative length of the linkage map and the average marker density were 6784 cM and 2.4 cM, respectively. We constructed a linkage map of 'KY08-129' using 1877 markers. These markers coalesced into 123 linkage groups (**Table 3**). The cumulative length of the linkage map and the average marker density were 5160.3 cM and 2.9 cM, respectively. We conducted composite interval mapping to identify QTLs for smut disease resistance using the linkage map of the parents, indices of smut disease resistance and polymorphism information of 4813 markers. Consequently, the identical LOD peak located at the end of linkage group 8 of 'Yaenoushie' was detected for three indices of smut disease incidence (**Fig. 3**). The highest LOD scores for percentage of infected plants in 2018, percentage of whip emergence in 2018 and percentage of whip emergence in 2019 were 45.6, 42.0 and 26.6, respectively (**Table 4**). This QTL explained 54% of infected plants in 2018, 53% of whip emergence in 2018 and 28.8% of whip emergence in 2019. The additive effects of this QTL for percentage of infected plants was –41.4%, while the percentage of whip emergence was –37.0% in 2018 and –16.6% in 2019. Half clones in the mapping population showed the genotype of 'Yaenoushie' at this QTL, and their percentages of whip emergence were 0% except for 3 clones.

In order to confirm if the QTL detected in the linkage map of 'Yaenoushie' is inherited from 'Iriomote8', we compared the genotype data of flanking markers among 'Yaenoushie', 'Iriomote8' and 'NiF8' (**Table 5**). Consequently, 'Yaenoushie' and 'Iriomote8' showed same genotypes, which were different from 'NiF8'.

Discussion

Construction of a high-density genetic map in sugarcane

Development of genetic maps in sugarcane is challenging mainly due to its highly polyploid and heterozygous characteristic. With the progress of genotyping technology, several genetic maps for sugarcane have been constructed (Aitken *et al.* 2014, Balsalobre *et al.* 2017). Yang *et al.* (2017) constructed the highest density linkage maps in sugarcane for QTL analysis using single-dose markers generated by genotyping-by-sequencing method. Single-dose markers effectively circumvent the complexity of polysomic inheritance. In this study, we constructed genetic maps of mapping parents, 'Yaenoushie' and 'KY08-129', using single-dose markers generated by GRAS-Di technology. This is the first report of linkage map construction in sugarcane using GRAS-Di technology. The number of linkage group of 'Yaenoushie' and 'KY08-129' were 117 and 123, respectively (**Tables 2**, **3**). This is highly consistent with the number of chromosomes of the sugarcane varieties previously reported ($2n = 100–130$). The average marker densities of our linkage maps were 2.4 cM for 'Yaenoushie' and 2.9 cM for 'KY08-129'. Although the average marker density of the high-density linkage maps of Yang *et al.* (2017) was 1.7 cM, our linkage maps showed a higher marker density compared to previous studies.

Detection of a major QTL related to smut disease resistance in sugarcane

There is a lack of reports on the detection of major QTLs

Table 2. Marker distribution in the linkage map of 'Yaenoushie'

Linkage group number	Number of markers	Total length (cM)	Linkage group number	Number of markers	Total length (cM)	Linkage group number	Number of markers	Total length (cM)
1	11	61.0	41	37	89.9	81	31	91.3
2	45	84.5	42	40	87.1	82	28	99.4
3	22	80.6	43	8	34.0	83	23	30.4
4	9	33.4	44	41	90.6	84	8	14.9
5	41	85.4	45	22	85.4	85	36	91.8
6	47	94.0	46	8	30.1	86	22	85.1
7	48	97.1	47	33	82.8	87	3	7.1
8	33	83.8	48	4	8.4	88	36	75.1
9	47	84.0	49	7	8.4	89	30	51.7
10	44	89.4	50	59	98.3	90	9	30.9
11	14	60.1	51	50	87.3	91	5	10.4
12	45	84.1	52	14	26.5	92	5	22.4
13	48	97.1	53	43	77.6	93	27	98.0
14	58	90.5	54	49	102.2	94	17	44.7
15	47	84.6	55	11	57.4	95	12	30.7
16	47	97.7	56	8	23.5	96	21	49.9
17	43	89.8	57	34	86.8	97	3	5.2
18	26	76.3	58	22	44.8	98	16	67.0
19	35	93.5	59	7	32.9	99	8	21.0
20	34	94.2	60	39	88.1	100	5	8.4
21	44	81.4	61	39	88.8	101	4	23.1
22	38	80.9	62	47	88.5	102	7	27.4
23	35	72.7	63	23	97.8	103	9	9.7
24	25	47.2	64	18	49.1	104	8	14.2
25	9	40.7	65	13	45.2	105	10	12.3
26	28	74.2	66	5	3.2	106	9	14.9
27	27	98.3	67	17	66.7	107	3	9.7
28	34	72.0	68	36	86.0	108	4	6.5
29	38	53.6	69	43	85.4	109	11	12.9
30	54	93.1	70	8	8.4	110	6	6.5
31	42	96.7	71	5	7.1	111	3	1.3
32	52	94.6	72	24	44.4	112	13	40.8
33	46	92.5	73	22	65.4	113	7	22.5
34	36	86.2	74	43	92.6	114	4	18.0
35	25	42.6	75	33	96.7	115	3	7.1
36	48	91.1	76	42	87.4	116	5	13.6
37	12	29.9	77	7	15.5	117	3	1.3
38	38	71.3	78	30	92.3	Total	2936	6784.0
39	25	76.2	79	40	87.5			
40	41	92.8	80	10	8.4			

in the sugarcane genome. Like most agronomic traits of sugarcane, smut disease resistance is thought to be a quantitative characteristic that is difficult to investigate genetically (Schaker *et al.* 2016). Our group identified highly smut disease resistant accessions of *S. spontaneum* collected in Japan, including 'Iriomote8' (Sakaigaichi *et al.* 2018). Using the mapping population derived from a resistant variety 'Yaenoushie', in which resistance is inherited from 'Iriomote8', we detected a major QTL related to smut disease resistance with a markedly high LOD score (**Fig. 3**, **Table 4**). To the best of our knowledge, this is the first report on the detection of a major QTL related to smut disease resistance in sugarcane. The distribution of disease incidence observed in this study tended to be "L shaped" (**Fig. 2**) and was similar to that of other sugarcane populations in which other major QTLs of sugarcane were detected (Aljanabi *et al.* 2007, Costet *et al.* 2012b, Daugrois *et al.* 1996). This indicates that there is a possibility of detecting a major QTL in sugarcane from a "L shaped" distribution of phenotype data.

An identical QTL was detected for percentage of whip emergence over two years, although the LOD score declined from 42.0 in 2018 to 26.6 in 2019. Comparing percentage of whip emergence in 2018 and 2019, the

Table 3. Marker distribution in the linkage map of 'KY08-129'

Linkage group number	Number of markers	Total length (cM)	Linkage group number	Number of markers	Total length (cM)	Linkage group number	Number of markers	Total length (cM)
1	44	83.4	42	36	81.1	83	30	68.1
2	21	53.1	43	7	7.8	84	7	16.2
3	25	69.0	44	30	58.5	85	6	7.8
4	14	23.9	45	5	23.4	86	10	22.8
5	24	106.0	46	5	6.5	87	9	26.6
6	13	23.3	47	3	1.9	88	21	62.2
7	24	89.9	48	39	90.6	89	12	34.7
8	19	24.6	49	14	50.4	90	3	1.3
9	53	101.8	50	21	72.8	91	7	27.3
10	14	62.6	51	13	25.2	92	7	5.8
11	13	35.6	52	12	14.9	93	10	18.7
12	21	51.1	53	9	35.3	94	24	51.8
13	11	18.8	54	32	98.5	95	9	84.0
14	31	96.1	55	8	13.6	96	9	26.0
15	13	46.2	56	10	30.4	97	5	9.1
16	19	44.0	57	35	72.5	98	3	1.9
17	32	79.0	58	36	92.4	99	19	85.9
18	24	86.0	59	12	35.1	100	6	7.1
19	24	59.0	60	41	76.3	101	11	17.4
20	40	98.8	61	7	10.3	102	4	5.8
21	36	96.2	62	22	57.9	103	8	25.3
22	19	48.9	63	11	18.8	104	18	82.8
23	4	7.8	64	9	38.7	105	7	49.1
24	17	51.1	65	12	34.1	106	4	17.9
25	15	19.4	66	12	20.7	107	11	34.4
26	24	42.0	67	15	53.6	108	11	70.3
27	12	41.7	68	18	42.0	109	4	2.6
28	25	99.0	69	11	29.1	110	6	41.6
29	11	7.1	70	6	5.8	111	4	18.9
30	5	29.7	71	11	35.3	112	9	24.1
31	10	34.1	72	10	74.7	113	4	9.7
32	3	3.2	73	4	8.4	114	6	11.6
33	18	57.4	74	37	81.5	115	5	12.3
34	28	88.3	75	11	15.5	116	5	15.5
35	17	42.7	76	13	13.6	117	3	1.9
36	19	51.9	77	33	89.3	118	7	15.6
37	23	98.3	78	18	60.6	119	4	3.9
38	26	60.0	79	15	75.0	120	5	12.3
39	24	99.4	80	12	34.5	121	3	20.3
40	11	21.3	81	21	65.6	122	4	35.2
41	24	37.5	82	8	25.4	123	3	8.4
						Total	1877	5160.3

disease incidence was lower in 2019 than in 2018 (**Fig. 2**). The most likely reason for this result is the reduction in the number of inoculated buds per clone from 40 buds in 2018 to 20 buds in 2019. Disease incidence can also fluctuate due to environmental conditions. Although future stabilization of the results is required, we believe the QTL detection, coupled with a remarkedly high LOD score (26.6 at lowest) at the same position, is a reliable result.

Evaluation of smut infection as an index of internal smut disease resistance

There are several reports on the evaluation of smut disease resistance in sugarcane by detecting the pathogen, *S. scitamineum*. Dalvi *et al.* (2012) detected smut infection in cane tops at 2 months after smut inoculation using PCR amplification and indicated that there was a high correlation between smut disease incidence in the field and PCR detection. Su *et al.* (2016) compared the copy number of *S. scitamineum* in inoculated buds by real-time PCR analysis

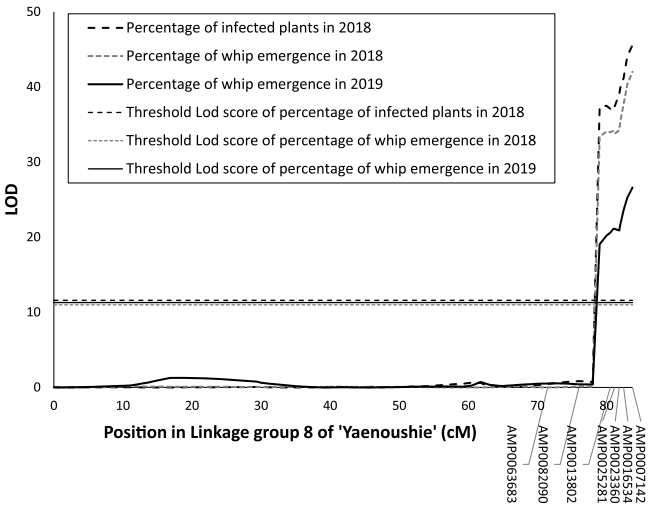

Fig. 3. LOD score profiles of linkage group 8 of 'Yaenoushie'.

and reported that the order of the amount of smut pathogens among varieties was similar to that of the resistant classification in the field. In this study, we detected smut infection in leaves at approximately 80 days after smut inoculation using PCR amplification. The percentage of infected plants was significantly correlated with percentage of whip emergence evaluated at approximately 200 days after smut inoculation (**Table 1**), and the identical QTL was detected for indices of smut disease incidence (**Fig. 3**). These results suggest that the detected QTL could be related to smut disease resistance during the early post-infection phase.

Smut infection into the bud meristem occurs between 6 and 36 hours after the teliospores deposition on buds (Alexander and Ramakrishnan 1980). Hyphae penetrates several bud scales to invade the bud meristem. External resistance is caused by differences in bud scale structure. In this study, we eliminated this resistance by inoculation with injection needles and evaluated internal resistance. Internal resistance is expressed after the pathogen penetrates through the bud scale. Que *et al.* (2014) compared gene expression following the wounding inoculation between 'Yacheng05-179' (resistant variety) and 'ROC22' (susceptible variety), and clarified that the largest difference occurred at 48 hours after inoculation compared with 24 and 120 hours after inoculation. Differentially expressed genes were involved in plant hormone signal transduction, flavonoid biosynthesis, plant-pathogen interaction, cell wall fortification pathway and other resistance-associated metabolic pathways. McNeil *et al.* (2018) conducted a similar analysis at 48 hours after inoculation using several varieties and detected differentially expressed genes involved in phenylpropanoid pathway, cell wall biosynthesis, plant hormone signal transduction and disease resistance genes. The accumulation of reactive oxygen species (ROS) in plant tissue is known to contribute to pathogen growth inhibition. Peters *et al.* (2017) observed that a resistant variety (SP80-3280) accumulated ROS in buds at 72 hours after inoculation. Su *et al.* (2016) suggested that smut disease resistance can be evaluated by measuring the activity of enzymes scavenging ROS. To elucidate the smut disease resistance mechanism inherited from 'Iriomote8', it is necessary to investigate the defense response of the resistant progeny from 0 to 80 days after inoculation. Furthermore, if the causal gene of the detected QTL is identified, it could be possible to clarify the key factors for smut disease resistance in sugarcane.

Table 5. Genotype of flanking markers of the QTL related to smut disease resistance

Markers	Linkage group	Position (cM)	Yaenoushie	NiF8	Iriomote8
AMP0063683	8	71.48	H[a]	A	H
AMP0082090	8	76.01	H	A	H
AMP0013802	8	80.54	H	A	H
AMP0025281	8	81.18	H	A	H
AMP0016534	8	82.47	H	A	H
AMP0007142	8	83.76	H	A	H

[a] H indicates the presence of marker. A indicates the absence of marker.

Smut disease resistance inherited from Japanese wild sugarcane

In this study, we detected a QTL related to smut disease resistance inherited from a Japanese *S. spontaneum* 'Iriomote8'. Smut disease resistant characteristics of *S. spontaneum* have been reported previously. Burner *et al.* (1993) evaluated the smut disease resistance of a United Sates Department of Agriculture germplasm collection using the wounding method and reported that there are several accessions of *S. spontaneum* that are highly resistant to smut disease. Modern sugarcane varieties are derived from a few interspecific hybridizations performed a century ago between *S. officinarum* and *S. spontaneum* (Berding and Roach 1987). Rody *et al.* (2019) compared the resistance gene analogs between different varieties showing different

Table 4. Detected QTL related to smut disease resistance

Traits	Linkage group	Position (cM)	Flanking marker	LOD scores	PVE[a] (%)	Additive effects	Threshold LOD scores	Genotype
Percentage of infected plants in 2018	8	83.8	AMP0007142	45.6	54.0	–41.4	11.6	Yaenoushie
Percentage of whip emergence in 2018	8	83.8	AMP0007142	42.0	53.0	–37.0	11.0	Yaenoushie
Percentage of whip emergence in 2019	8	83.8	AMP0007142	26.6	28.8	–16.6	11.3	Yaenoushie

[a] PVE indicates the percentage of phenotype variance explained by each QTL.

smut disease resistances. Their result indicated that smut disease resistance genes in modern sugarcane varieties originated in chromosome 5 of the ancestral *S. spontaneum* genotype. Zhang *et al.* (2018) published the genome of *S. spontaneum* 'AP85-441' (2n = 4x = 32) generated from a culture of 'SES208' (2n = 8x = 64). They confirmed that 80% of 361 putative disease resistance genes were located in the genome of 'AP85-441'. Taking the above into consideration, the high smut disease resistant characteristic of *S. spontaneum* might not be specific to Japanese accessions. To identify the causal gene of smut disease resistance analyzed in this study, a homology search between the genome of 'AP85-441' and the linkage map of 'Iriomote8' will be an effective tool. We are developing reliable DNA markers linked to the identified QTL to conduct MAS for smut disease resistance in our sugarcane breeding program. Confirmation of the existence of these DNA markers in our germplasm collection is also an important future consideration.

In conclusion, we detected a QTL for smut disease resistance inherited from a Japanese wild sugarcane, 'Iriomote8', with a markedly high LOD score. Furthermore, there were no correlations between smut disease resistance and other agronomic traits in the mapping population. This QTL could be used for MAS to obtain significant improvements in smut disease resistance without negative effects on other agronomic traits.

Author Contribution Statement

T.S., M.T. and Y.T. developed the plant material and designed the study. M.U. obtained phenotype data. T.K. and M.M. obtained genotype data. M.U., T.K. and M.M. conducted the data analysis. A.K., T.H., M.H., H.T. and Y.T. provided scientific advice to the corresponding author. M.U. wrote the paper.

Acknowledgments

We are grateful to Okinawa Prefectural Agricultural Research Center for transferring teliospores used in this study. We appreciate the technical support of Y. Oitate, M. Habu, H. Nagano, M. Nishikawa, K. Matsugami, T. Miike, M. Kubo and contract staff of Tanegashima sugarcane breeding site including N. Takei.

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