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Research Paper

DNA marker for resistance to *Puccinia horiana* in chrysanthemum (*Chrysanthemum morifolium* Ramat.) "Southern Pegasus"

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White rust caused by *Puccinia horiana* Henn. adversely affects chrysanthemum (*Chrysanthemum morifolium* Ramat.) production. The breeding of resistant varieties is effective in controlling the disease. Here we aimed to develop DNA markers for the strong resistance to *P. horiana*. We conducted a linkage analysis based on the genome-wide association study (GWAS) method. We employed a biparental population for the GWAS, wherein the single nucleotide polymorphism (SNP) allele frequency could be predicted. The population was derived from crosses between a strong resistant "Southern Pegasus" and a susceptible line. The GWAS used simplex and double-simplex SNP markers selected out of SNP candidates mined from ddRAD-Seq data of an F_1 biparental population. These F_1 individuals segregated in a 1:1 ratio of resistant to susceptible. Twenty-one simplex SNPs were significantly associated with *P. horiana* resistance in "Southern Pegasus" and generated one linkage group. These results show the presence of a single resistance gene in "Southern Pegasus". We identified the nearest SNP marker located 2.2 cM from *P. horiana* resistance locus and demonstrated this SNP marker-resistance link using an independent population. This is the first report of an effective DNA marker linked to a gene for *P. horiana* resistance in chrysanthemum.

Key Words: chrysanthemum white rust, genome-wide association study, simplex, single nucleotide polymorphism marker.

Introduction

Chrysanthemum white rust is an important disease of chrysanthemums, *Chrysanthemum morifolium* Ramat., in Japan. It is caused by *Puccinia horiana* Henn., first detected in Japan in 1895 (Baker 1967, Hiratsuka 1957), and currently widespread throughout the world (O'Keefe and Davis 2015). *P. horiana* is an autoecious microcyclic rust fungus with a life cycle that is completed on a single host and involves two spore stages; teleutospores are not released but germinate to produce basidiospores under highly humid conditions. These basidiospores are dispersed by air currents, and thus re-infect chrysanthemum leaves under conditions that are highly humid or wet (Firman and Martin 1968, Yamada 1956). *P. horiana* infects many chrysanthemum species (Hiratsuka 1957, Park *et al.* 2014, Yamaguchi 1981, Zeng *et al.* 2013), including the cultivated chrysanthemum which is one of the most important ornamental plants worldwide, providing cut flowers and both potted and garden plants. *P. horiana* is a major pathogen of cultivated chrysanthemum and has been reported in most growing areas, forming raised buffs or pinkish pustules mainly on the lower leaf surface. *P. horiana* causes significant economic losses in commercial production. Chemical control has become difficult due to an increasing number of fungicide-resistant isolates (Cook 2001) and a decreasing number of registered fungicides. In addition, environmental control, consisting of lowering the relative humidity, is not always feasible, such as in open-fields and semi-covered growing structures. One of the most effective methods of disease control is the use of resistant cultivated varieties. Resistant chrysanthemum cultivars have been well-studied (Baker 1967, de Backer *et al.* 2011, Dickens 1968, Martin and Firman 1970, Park *et al.* 2014, Yamaguchi 1981), including the inheritance of *P. horiana*

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resistance; such studies suggest that most of resistant cultivars carry a single dominant gene (De Jong and Rademaker 1986). Thus far, however, no DNA marker associated with *P. horiana* resistance has been described. Such markers are useful for marker-assisted selection (MAS) in *P. horiana*-resistance breeding.

MAS efforts have lagged behind because of the complicated segregation pattern of markers that result from the autohexaploid genome of cultivated chrysanthemum (2n = 6x = 54); (van Geest *et al.* 2017b). This complex genome can produce a total of seven different allele patterns (AAAAAA, AAAAAa, AAAAaa, AAAaaa, AAaaaa, Aaaaaa, and aaaaaa) at a single locus, assuming monogenic inheritance on a locus with two alleles (e.g., A vs a). Thus, in a cross between a heterozygous parent and a recessive-homozygous parent (aaaaaa), the expected segregation ratios depend on the heterozygous allele pattern. To develop DNA markers for cultivated chrysanthemum, specialized methods for linkage mapping and QTL analysis are needed (van Geest *et al.* 2017a). Although GWAS has traditionally been used to analyze broad, diverse populations, we recently reported a straightforward GWAS-based system for developing markers in chrysanthemum, in which a biparental population was employed for predicting the SNP allele frequency and no novel statistics were available (Sumitomo *et al.* 2019). Our approach used next-generation sequencing technology, thus enabling a comprehensive and efficient analysis of DNA markers in chrysanthemum. Here, we report the development of such DNA markers for *P. horiana* resistance in chrysanthemum.

Materials and Methods

Plant materials and DNA extraction

Our F_1 population originated from a cross between susceptible "NARO_cgs0302033" and resistant "Southern Pegasus" (**Fig. 1**). A total of 128 F_1 seedlings were planted in plastic pots (12-cm internal diameter, one seedling per pot) containing a commercial horticultural soil (Kureha-Engei-Baido; Kureha Chemical Co. Ltd., Tochigi, Japan) and maintained in the vegetative state as mother plants in a glasshouse maintained between 18°C and 25°C and 6-h night-break conditions. Genomic DNA was extracted from the shoot tips (30 mg fresh weight) using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Phenotyping of plants susceptible/resistant to Puccinia horiana

Diseased leaves containing *P. horiana* were collected from "Floral Yuka" plants grown at Institute of Vegetable and Floriculture Science, NARO (Tsukuba, Japan) in 2017 and used as a source of inoculum. A single pustule culture of the isolate was established and maintained on fresh and *P. horiana*-free cuttings of "Shuho-no-chikara" as described by Alaei *et al.* (2009).

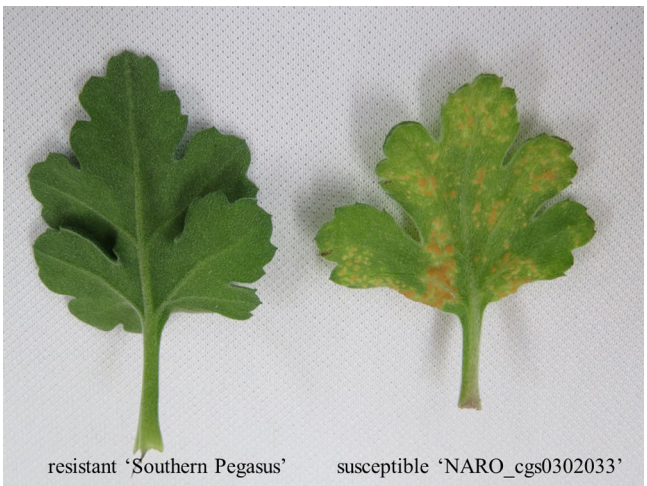

Fig. 1. Abaxial side of leaves of susceptible "NARO_cgs0302033" and resistant "Southern Pegasus" cultivars at 28 days after *P. horiana* inoculation.

Assays were conducted using a Styrofoam box (50.8 cm internal length × 36.0 cm internal width × 34.9 cm internal depth) in a growth chamber. Fresh cuttings from the F_1 population and parents (one cutting per line) were inserted in a 200-well cell tray containing a horticulture medium (Metro Mix 360; Scotts Co., Marysville, OH, USA) and placed on the bottom of a Styrofoam box. A plastic net (5-mm mesh) covered the top opening of the box. Inoculum was prepared from fresh cuttings of infected "Shuho-no-chikara" as described by de Backer *et al.* (2011). Heavily-infected leaves were collected and cut into approximately 1-cm^2 pieces. These fragments were distributed at a density of 3 cm × 3 cm with their telia pointing downwards on the net. To ensure high relative humidity and water film on the leaves, the cuttings, inner sides of the box and the net holding the inoculum were misted with demineralized water using a sprayer. The box was closed and placed in a dark growth chamber at 19°C. Sixteen hours after the start of the inoculation, the 200-well cell tray was transferred into a plastic transparent container (37.5 cm internal length × 24.7 cm internal width × 12.9 cm internal height) and placed in a growth chamber maintained at 22°C with a 16-h photoperiod provided by fluorescent white-light tubes (100 µmol m^{-2} s^{-1}; FHF32EX-N-HG; NEC Co., Tokyo, Japan). Symptoms were evaluated 28 days after inoculation.

The assay was conducted three times. Three follow-up assays were conducted on F_1 plants showing no visible disease symptom in the first round of the three assays. If at least one pustule on a plant was observed in any of the assays, a phenotype of "susceptible (S)" was given. Plants showing no teliospores throughout six assays were scored "resistant (R)".

ddRAD-Seq analysis

Genomic DNA from the F_1 population and its parental

lines was double-digested with *PstI* and *MspI* to generate ddRAD-Seq libraries, as described in Shirasawa *et al.* (2017). Nucleotide sequences of the libraries were determined on a HiSeq4000 (Illumina) platform in paired-end, 101-bp mode.

Data processing and simplex single nucleotide polymorphism (SNP) mining

Data processing of sequence reads and simplex SNP calling were performed as described by Sumitomo *et al.* (2019). In brief, sequence reads obtained from ddRAD-Seq analysis were mapped onto the *C. seticuspe* genome sequence (CSE_r1.0) (Hirakawa *et al.* 2019), used as a reference. High-confidence SNP candidates were mined and called from the resulting sequence alignments using the following criteria: (i) depth of coverage of sequence reads is ≥10 for each data point and (ii) proportion of missing data is <0.25 for each locus.

In diploid species, read counts of the pooled progeny's samples have been used at each SNP locus to estimate the genotypes of the parental lines (Ashraf *et al.* 2014). This approach is also effective in hexaploid species (Shirasawa *et al.* 2017). We selected simplex SNPs, "AAAAAA × AAAAAa" and "Aaaaaa × aaaaaa", and double-simplex SNPs, "AAAAAa × AAAAAa" and "Aaaaaa × Aaaaaa", according to the alternative allele frequency (AAF) of the pooled F_1 progeny's samples at each SNP locus. The AAF for each position was calculated by dividing the number of reads with variant-supporting bases by the number of total reads aligned at the position.

Simplex SNP sites of "AAAAAA × AAAAAa (AAF = 1/12 = 0.083)" and "Aaaaaa × aaaaaa (AAF = 11/12 = 0.917)" were selected for cases in which the AAF value is ≥0.042 and <0.125 and those in which AAF ≥0.875 and <0.958, respectively. Double-simplex SNP sites of "AAAAAa × AAAAAa (AAF = 2/12 = 0.167)" and "Aaaaaa × Aaaaaa (AAF = 10/12 = 0.833)" were selected for cases in which the AAF value is ≥0.125 and <0.208 and those in which AAF ≥0.792 and <0.875, respectively. Furthermore, the genotype of each individual was determined based on genotypes of F_1 individuals of the SNP loci. Theoretically, the "AAAAAa × AAAAAa" double-simplex SNPs would be expected to segregate into AAAAAA (AAF = 0/6 = 0.000), AAAAAa (AAF = 1/6 = 0.167) and AAAAaa (AAF = 2/6 = 0.333) at a ratio of 1:2:1 in the F_1 progeny. However, it was difficult to distinguish between the AAAAAa and AAAAaa genotypes because the numbers of reads of each individual were insufficient to clearly differentiate between AAFs of 0.167 and 0.333. Therefore, AAFs of 0 and >0.000 were scored as homozygous (0/0) and nonhomozygous reference alleles (0/1), respectively, with an expected segregation ratio of 1:3, as with dominant loci. Correspondingly, AAFs of 1 and <1.000 were encoded as homozygous (1/1) and nonhomozygous alternative alleles (0/1), respectively, for following GWAS. In addition, subsets of segregation data of double-simplex and simplex loci that fitted the expected ratio of 3:1 and 1:1, respectively, were selected based on chi-square tests ($P > 0.01$).

Genome-wide association study

Associations between genotypes and phenotypes were analyzed with a general linear model using the TASSEL program (Bradbury *et al.* 2007) with the default parameters. The thresholds for the association were set at 8.7×10^{-8} (=0.001/11515) at a significance level of 0.1% after implementing the Bonferroni multiple test correction (Benjamini and Hochberg 1995).

SNP marker associated with P. horiana resistance

We investigated SNP-distinguishable PCR-based markers for the SNPs associated with *P. horiana* resistance. Allele-specific primers were designed corresponding to the SNPs (**Supplemental Table 1**) by browsing sequence reads using IGV software (Robinson *et al.* 2011). The SNP was validated by PCR using 8 ng of genomic DNA from the parents and F_1 individuals. Touchdown PCR was performed with the following conditions: 95°C for 50 s, 40 cycles of 95°C for 5 s, annealing for 15 s, and 72°C for 20 s, where the annealing temperature is gradually reduced 2°C every third cycle from the initial annealing temperature of 66°C to the final annealing temperature 56°C. PCR was performed using the TB Green Premix Ex Taq II Tli RNase H plus kit (TaKaRa Bio, Shiga, Japan) on a thermal Cycler Dice Real-Time system (TaKaRa Bio).

Linkage analysis

Linkage analysis was performed by JoinMap® 4.1 software (Kyazma B.V., the Netherlands). The PCR genotype data of the SNP markers associated with *P. horiana* resistance was imported into the software program along with the phenotype data of the qualitative trait for *P. horiana* resistance in F_1 population. The BC1 population option was used for data mining, based on LOD threshold of 10.0. The map was constructed using default regression mapping parameters and the Kosambi mapping function was used for the calculation of the genetic distance between markers.

Validation of SNP marker-resistance link in an independent population

We prepared a population of 63 F_1 plants originating from a cross between "Yellow Queen" (susceptible) and "Southern Pegasus" to investigate whether the SNP marker is valid in another independent population. We evaluated this population for *P. horiana* resistance and genotyped the plants using the resistance-linked SNP marker, SCSE_SC008866.1_53841.

Results

Phenotype data

Symptoms were observed 14 days after the start of inoculation (dpi) and were easy to evaluate at 21 dpi, but a final

Table 1. General linear model association analysis showing 22 single nucleotide polymorphisms associated with *P. horiana* resistance

SNP marker name	*P* value	Major allele	Minor allele	Parental genotype		Number of F_1 individuals of marker genotype in ddRAD-Seq data			Average sequence read coverage depth	Reads number	Alternative allele frequency
				Southern Pegasus	NARO_cgs0302033	Heterozygote	Homozygote	Missing data			
SCSE_SC004988.1_69310	1.94×10^{-42}	G	A	GGGGGA	GGGGGG	70	54	4	88.6	10990	0.060
SCSE_SC023417.1_15736	2.85×10^{-31}	G	A	GGGGGA	GGGGGG	73	51	4	68.1	8447	0.118
SCSE_SC055248.1_9363	3.15×10^{-29}	T	C	TTTTTC	TTTTTT	74	50	4	95.6	11860	0.064
SCSE_SC003052.1_70044	1.08×10^{-25}	A	T	AAAAAT	AAAAAA	76	48	4	220.0	27260	0.114
SCSE_SC021297.1_19351	1.33×10^{-25}	G	C	GGGGGC	GGGGGG	70	54	4	115.1	14275	0.077
SCSE_SC004314.1_12261	4.34×10^{-24}	A	G	AAAAAG	AAAAAA	56	49	23	42.8	4499	0.052
SCSE_SC018378.1_31351	9.68×10^{-24}	C	A	CCCCCA	CCCCCC	68	55	5	57.5	7074	0.057
SCSE_SC003182.1_58579	2.18×10^{-23}	A	C	AAAAAC	AAAAAA	61	52	15	43.7	4941	0.100
SCSE_SC002003.1_88623	1.04×10^{-18}	G	A	GGGGGA	GGGGGG	53	61	14	44.3	5047	0.109
SCSE_SC005358.1_66101	4.45×10^{-17}	G	A	GGGGGA	GGGGGG	53	59	16	64.4	7218	0.088
SCSE_SC004314.1_12293	5.68×10^{-17}	T	C	TTTTTC	TTTTTT	62	43	23	39.2	4115	0.066
SCSE_SC003052.1_70007	7.20×10^{-17}	G	A	GGGGGA	GGGGGG	58	66	4	219.8	27258	0.046
SCSE_SC001398.1_54103	3.51×10^{-15}	G	A	GGGGGA	GGGGGG	72	51	5	125.3	15416	0.092
SCSE_SC000596.1_124136	3.53×10^{-14}	G	C	GGGGGC	GGGGGC	88	35	5	135.1	16612	0.173
SCSE_SC008866.1_53841	4.89×10^{-13}	A	G	AAAAAG	AAAAAA	56	61	11	38.1	4459	0.091
SCSE_SC000727.1_69385	6.05×10^{-10}	A	T	AAAAAT	AAAAAA	44	67	17	44.1	4891	0.075
SCSE_SC000727.1_69400	1.14×10^{-9}	T	C	TTTTTC	TTTTTT	44	68	16	44.7	5006	0.074
SCSE_SC024966.1_20397	1.72×10^{-9}	T	C	TTTTTC	TTTTTT	65	53	10	61.3	7238	0.101
SCSE_SC024966.1_20411	2.69×10^{-9}	A	T	AAAAAT	AAAAAA	62	57	9	60.9	7252	0.100
SCSE_SC001915.1_65335	3.33×10^{-9}	C	T	CCCCCT	CCCCCC	73	50	5	56.2	6910	0.119
SCSE_SC000727.1_69352	4.24×10^{-9}	T	A	TTTTTA	TTTTTT	45	66	17	44.0	4879	0.075
SCSE_SC021342.1_22044	8.36×10^{-9}	C	T	CCCCCT	CCCCCC	73	51	4	102.6	12728	0.119

evaluation was done at 28 dpi. One hundred and twenty-eight F_1 individuals of a cross between *P. horiana*-susceptible "NARO_cgs0302033" and -resistant "Southern Pegasus" segregated in a 73:55 ratio of R to S (**Supplemental Table 2**). This roughly fits the expected 1:1 ratio ($\chi^2 = 2.53$, $P = 0.11$) of a simplex × nulliplex (Aaaaaa × aaaaaa) cross for hexasomic inheritance. This also indicates that "Southern Pegasus" has a single dominant gene for *P. horiana* resistance.

GWAS for P. horiana resistance

Approximately 2.9 M high-quality reads per sample were obtained from the F_1 population (n = 128) and parental cultivars. Maximum reads per sample, minimum reads per sample, and standard deviation in the samples were 6,393,787, 80,489, and 1,285,780, respectively. The sequence reads were registered in Sequence Read Archive database in DNA Data Bank of Japan (accession number DRA010049). Of the sequence reads, 80.1% were mapped on the reference *C. seticuspe* genome, CSE_r1.0 (Hirakawa *et al.* 2019). A total of 73,338 high-confidence, SNP candidates were identified. Of these, 2,446 double-simplex (Aaaaaa × Aaaaaa or AAAAAa × AAAAAa) and 9,069 simplex (Aaaaaa × aaaaaa, aaaaaa × Aaaaaa, AAAAAa × AAAAAA, or AAAAAA × AAAAAa) SNPs that significantly fit the expected 3:1 and 1:1 ratios, respectively, were selected in accordance with the AAF scores and chi-square tests ($P > 0.01$).

GLM analysis of 11,515 (i.e., 2,446 + 9,069) SNP markers identified 22 SNP markers (21 simplex and 1 double-simplex SNPs) that were significantly associated with *P. horiana* resistance (**Table 1**). However, the single double-simplex SNP, SCSE_SC000596.1_124136, was an error because it was the result of an incorrect alignment of sequence reads mapped onto the reference *C. seticuspe* genome. SNP marker SCSE_SC004988.1_69310 showed the highest association, with the lowest *P* value of 1.94×10^{-42}. The ddRAD-Seq results showed that the genotypes of the SNP marker, SCSE_SC004988.1_69310 in resistant "Southern Pegasus" were heterozygous for GGGGGA, whereas those in susceptible "NARO_cgs0302033" were homozygous for GGGGGG. SCSE_SC000727.1_69352, 69385 and 69400, SCSE_SC003052.1_70007 and 70044, SCSE_SC024966.1_20397 and 20411, respectively, were located on the same sequence by browsing sequence reads using IGV software.

Linkage analysis

Genetic linkage group was constructed (**Fig. 2**) using the PCR genotype data (**Supplemental Table 2**) for the SNP markers associated with *P. horiana* resistance. The simplex SNPs generated one linkage group. This shows that these SNP markers are genetically linked. Among the 16 contigs within which the 21 SNPs are located, two sequences (SCSE_SC000727.1 and SCSE_SC002003.1) reside on linkage group 6 of the *C. seticuspe* linkage maps (Hirakawa *et al.* 2019); the other sequences have not been assigned to any place on the map. The genetic locus of *P. horiana*

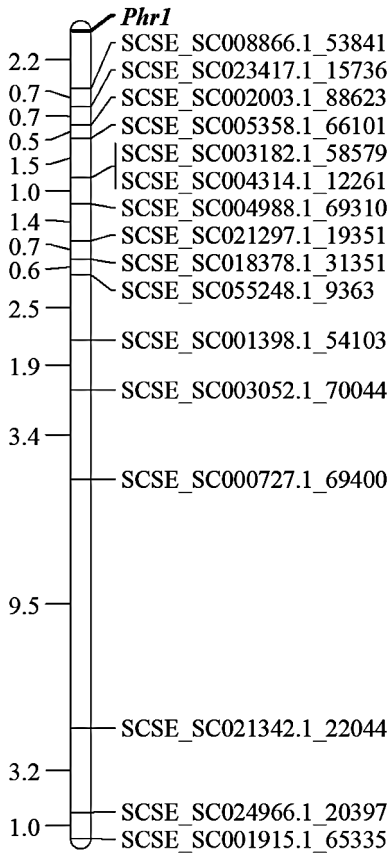

Fig. 2. Genetic linkage map with the simplex single nucleotide polymorphisms (SNPs) associated with *P. horiana* resistance in "Southern Pegasus" (*Phr1*). SNP loci are shown on the right side of linkage group. Numbers on the left side indicate genetic distances (cM).

resistance (*Phr1*, *P. horiana* resistance locus 1) was located at the end of linkage group, on 2.2 cM from the nearest SNP, SCSE_SC008866.1_53841. The genotype of SCSE_SC008866.1_53841 in resistant "Southern Pegasus" was heterozygous for AAAAAG, whereas in susceptible "NARO_cgs0302033" was homozygous for AAAAAA. The G allele of SNP and *Phr1* locus was in the coupling phase in the "Southern Pegasus" genome. The SNP marker SCSE_SC004988.1_69310 showing the highest association in GWAS was not the flanking marker and was located 6.6 cM from *Phr1*.

In the 128 F_1 plants, segregation of the homozygous (AAAAAA):heterozygous (AAAAAG) in the flanking marker SCSE_SC008866.1_53841 was 54:74 (**Table 2**), roughly fitting the 1:1 segregation ratio for a simplex × nulliplex (1 × 0) cross for hexasomic inheritance. Seventy-one F_1 plants carrying the G allele of the resistant parent exhibited *P. horiana* resistance; three F_1 plants that were susceptible indicating that these plants do not carry the *Phr1* allele. Fifty-two F_1 plants homozygous for the A alleles were susceptible. But two F_1 plant exhibited resistance. These results demonstrate the recombination between the G allele on SCSE_SC008866.1_53841 and *Phr1* in the five plants.

Table 2. Relationship between marker genotype linked to *Phr1* and *P. horiana* resistance in 128 F_1 plants

Marker genotype of SCSE_SC008866.1_53841	*P. horiana* resistance	
	Resistant	Susceptible
AAAAAA	2	52
AAAAAG	71	3

Table 3. Relationship between marker genotype and *P. horiana* resistance in a population from a cross between susceptible "Yellow Queen" and "Southern Pegasus"

Minor (G) allele of SCSE_SC008866.1_53841	*P. horiana* resistance	
	Resistant	Susceptible
Presence	31	0
Absence	1	31

Validation of the SNP marker-resistance link in an independent population

We tested the SNP marker link to disease resistance in a population resulting from a cross between susceptible "Yellow Queen" and "Southern Pegasus". PCR analysis using allele-specific primers showed amplification of the A allele but not the G allele for SCSE_SC008866.1_53841 in "Yellow Queen" (data not shown). This result indicates that the six alleles of autohexaploid contained at least one A allele and no G allele for SCSE_SC008866.1_53841, but the exact allele pattern is not known. Thus, we investigated the presence or absence of the G allele from "Southern Pegasus" in this experiment. The segregation of *P. horiana* resistance in this population was at 32:31 ratio of R to S (**Table 3**). This agrees with the expected 1:1 segregation ($\chi^2 = 0.02$, $P = 0.90$). Every F_1 plant carrying the G allele of the resistant "Southern Pegasus" exhibited *P. horiana* resistance. Of 32 F_1 plants without the G allele, 31 F_1 plants were susceptible and 1 F_1 plants were resistant. The SNP marker SCSE_SC008866.1_53841 was useful in an independent population.

Discussion

De Jong and Rademaker (1986) described the following three types of resistance to *P. horiana* in chrysanthemum cultivars: 1. Complete resistance, where no symptoms visible, and no spore production; 2. incomplete resistance, where few pustules develop slowly and produce a limited number of spores; and 3. necrosis, where necrotic areas develop around the growing rust colonies and sporulation may not be completely inhibited. In fact, to a large extent, necrosis inhibits spore formation. The resistance of "Southern Pegasus" is classified as "complete" because the plant did not show any visible symptoms throughout the experiments (**Fig. 1**). De Jong and Rademaker (1986) reported that "completely resistant" cultivars carry a single dominant gene, mostly in a simplex. Thus, we expected "Southern

Pegasus" to have a single dominant resistance gene that is inherited qualitatively. Therefore, we used a simple scoring system of R or S in this study, although previous reports scored the phenotype quantitatively, i.e., by the relative leaf area covered with teliospores (Takatsu *et al.* 2000, Yamaguchi 1981).

Even though autohexaploid cultivated chrysanthemums have complicated segregation patterns, the segregation ratio of resistance versus susceptible follows a relatively simple 1:1 ratio in the F_1 population, as this study clearly shows (**Tables 2**, **3**). Thus, resistance in "Southern Pegasus" is monogenic inheritance, that is, "Southern Pegasus" has a single resistance gene. This was confirmed by results from GWAS and linkage analysis (**Table 1**, **Fig. 2**). The GWAS approach was designed to detect associations between DNA markers and causal genes based on linkage disequilibrium (Yu *et al.* 2006). In this study using a biparental population, we assumed that 21 simplex SNPs detected by GWAS are related via haplotype block in the "Southern Pegasus" genome. Linkage analysis shows that the simplex SNPs associated with *P. horiana* resistance in "Southern Pegasus" generated one linkage group (**Fig. 2**), suggesting that the plant has a single resistance gene in its genome. Results of linkage analysis, as well as the 1:1 segregation ratio, clearly identify a single resistance gene in "Southern Pegasus". Linkage analysis also guarantees that the 21 simplex SNPs did not contain false positives, which is an issue because it has been reported that GWAS based on GLM may generate many false positives (Hwang *et al.* 2014, Sun *et al.* 2016).

Genotyping by PCR and linkage analysis showed that the nearest neighbor of *Phr1* was SCSE_SC008866.1_53841 (**Fig. 2**), but this SNP marker was relatively less significant in the GWAS results (**Table 1**). The sequence read depth of SCSE_SC008866.1_53841 was the lowest among all SNPs, which may have resulted in relatively low genotyping accuracy in ddRAD-Seq analysis. We did not identify any markers on the upper side of *Phr1* (**Fig. 2**), which may be located at the chromosomal end.

Two sequences (SCSE_SC000727.1 and SCSE_SC002003.1) on the haplotype block harboring *Phr1* reside on linkage group 6 of the *C. seticuspe* linkage maps (Hirakawa *et al.* 2019). This indicates that the location of the *Phr1* gene on the "Southern Pegasus" chromosome may correspond to linkage group 6 of *C. seticuspe*, although chromosomal collinearity is still unclear between these two species. Unfortunately, the sequence of the genome of *C. seticuspe* is at the draft stage and is highly fragmented at present (Hirakawa *et al.* 2019). Therefore, it is impossible to generate a Manhattan plot demonstrating the location of associated SNPs along the chromosomes; and it is also impossible to define a candidate region within a single contiguous sequence. Prior to this study, we had neither the information on the physical distances between SNPs nor the candidate genes within this interval. Currently, we are improving the genome assembly of *C. seticuspe* to obtain the sequence spanning the candidate region.

Plants have evolved sophisticated resistance systems against pathogens. In turn, pathogens have also evolved features for the evasion of plant resistance systems. This interaction between plants and pathogens has resulted in plants gaining a number of resistance genes, as proposed in the gene-for-gene hypothesis (Flor 1956, 1971). For example, 60 genes in wheat are responsible for resistance to leaf rust caused by *Puccinia triticina* Eriks. (Bolton *et al.* 2008). Moreover, a number of resistance genes in chrysanthemum may be inferred because many races of *P. horiana* have been identified (de Backer *et al.* 2011, Yamaguchi 1981). Furthermore, resistant cultivars or accessions have been reported (de Backer *et al.* 2011, De Jong and Rademaker 1986, Yamaguchi 1981). Therefore, combining multiple race-specific resistance genes, using partial-resistance genes, or pyramiding of both into a single plant are desirable goals for breeding durable *P. horiana*-resistant chrysanthemum cultivars. If molecular markers tightly linked to the target genes are available, then pyramiding resistance genes by MAS may speed up the development of resistant cultivars. Toward this goal, this study presents the first report of a DNA marker for *P. horiana* resistance in chrysanthemum. Our results will accelerate the development of *P. horiana*-resistant cultivars.

Author Contribution Statement

KS designed and executed the study, prepared all tables and figures, and wrote the manuscript. KS helped with ddRAD-Seq analysis and GWAS. AH conducted a part of inoculation test. SI, HH, MK, MY, MO, MK, and FT contributed to data analysis and corrections in the manuscript.

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