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

Katsuhiko Sumitomo*1), Kenta Shirasawa3), Sachiko N. Isobe2), Hideki Hirakawa2), Akiho Harata1,4), Masato Kawabe1), Masafumi Yagi1), Masaaki Osaka5) and Fumiya Taniguchi6)

1) Institute of Vegetable and Floriculture Science, NARO, Tsukuba, Ibaraki 305-0852, Japan
2) Kazusa DNA Research Institute, Kisarazu, Chiba 292-0818, Japan
3) Kagoshima Prefectural Institute for Agricultural Development, Minamiasa, Kagoshima 899-3401, Japan
4) Present address: CCS Inc., Kyoto 602-8019, Japan
5) Miyagi Prefectural Institute of Agriculture and Horticulture, Natori, Miyagi 981-1243, Japan
6) Institute of Fruit Tree and Tea Science, NARO, Tsukuba, Ibaraki 305-8605, Japan

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 autecious 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*.
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 (AAAAAAA, AAAAAa, AAAAaa, AAAAAa, AAAAA, Aaaaaa, Aaaa, 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 (aaaaaaa), 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 F1 population originated from a cross between susceptible “NARO_cgs0302033” and resistant “Southern Pegasus” (Fig. 1). A total of 128 F1 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).

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 F1 population and parents (one cutting per line) were inserted in a 200-cell well 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-cm2 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-cell well 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 F1 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 F1 population and its parental...
lines was double-digested with *Pst*I and *Msp*I 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 F1 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 F1 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 AAAAAaa (AAF = 2/6 = 0.333) at a ratio of 1:2:1 in the F1 progeny. However, it was difficult to distinguish between the AAAAAa and AAAAAaa 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^{-4} (=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 F1 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 F1 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 F1 plants originating from a cross between “Yellow Queen” (susceptible) and “Southern Pegasus” to investigate whether the SNP marker resistance was imported into the software program along with the phenotype data of the qualitative trait for *P. horiana* resistance in F1 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.

*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
evaluation was done at 28 dpi. One hundred and twenty-eight F1 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 hexagonal 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 F1 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. SNP marker SCSE_SC004988.1_69310 showed the highest association, with the lowest $P$ value of 1.94 × 10⁻⁴. 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_SC007271.1_69352, 69385 and 69400, SCSE_SC000727.1_69385 and 69400, SCSE_SC000727.1_69385 showed the highest association, with the lowest $P$ value of 1.94 × 10⁻⁴. 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_SC007271.1_69352, 69385 and 69400, SCSE_SC000727.1_69385 and 69400, SCSE_SC000727.1_69385 showed the highest association, with the lowest $P$ value of 1.94 × 10⁻⁴.

**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...
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 F1 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 F1 plants carrying the G allele of the resistant parent exhibited P. horiana resistance; three F1 plants that were susceptible indicating that these plants do not carry the Phr1 allele. Fifty-two F1 plants homozygous for the A alleles were susceptible. But two F1 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 F1 plants

| Marker genotype of SCSE_SC008866.1_53841 | P. horiana resistance |
|-----------------------------------------|-----------------------|
| AAAAAG                                  | 2                     |
| AAAAAG                                  | 52                    |

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 F1 plant carrying the G allele of the resistant “Southern Pegasus” exhibited P. horiana resistance. Of 32 F1 plants without the G allele, 31 F1 plants were susceptible and 1 F1 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 F1 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_SC00727.1 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 Redemaker 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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