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A genome-wide association and fine-mapping study of white rust resistance in hexaploid chrysanthemum cultivars with a wild diploid reference genome

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Abstract

White rust caused by Puccinia horiana is one of the most serious diseases of chrysanthemum (Chrysanthemum × morifolium). In this study, we report the DNA markers associated with resistance against P. horiana via a simple approach using the genome of a wild diploid relative, Chrysanthemum seticuspe. First, we identified the important region of the genome in the resistant cultivar “Ariesu” via a genome-wide association study. Simplex single nucleotide polymorphism (SNP) markers mined from ddRAD-Seq were used in a biparental in F1 progenies originating from resistant “Ariesu” and showed robust transferability for detecting markers that were closer to those identified by comparing the assembled genome sequences of resistant “Ariesu” and susceptible “Yellow Queen”. Consequently, SNP markers that were closer to Phr2 compared with ddRAD-Seq markers were obtained. These SNP markers co-segregated with resistance in F1 progenies originating from resistant “Ariesu” and showed robust transferability for detecting Phr2-conferring resistance among chrysanthemum genetic resources. The wild C. seticuspe pseudomolecule, a de facto monoploid genome used for ddRAD-Seq analysis and assembled genome sequence comparison, demonstrated this method’s utility as a model for developing DNA markers in hexaploid chrysanthemum cultivars.

Introduction

Chrysanthemum (Chrysanthemum × morifolium Ramat.) is an important ornamental crop worldwide. White rust is a major disease caused by Puccinia horiana Henn. in chrysanthemums. It was first detected in Japan in 1895 [3, 21]. Since then, it has spread worldwide [35]. P. horiana forms raised blisters or pink pustules primarily on the lower surface of the leaves, causing major economic losses in commercial production. One of the most effective disease control methods is the use of P. horiana-resistant cultivars. Several P. horiana-resistant chrysanthemum cultivars have been reported [3, 7–9, 31, 38, 51]. Therefore, developing resistance-linked DNA markers will contribute to efficient resistance breeding.

To date, DNA marker development for white rust has been challenging because chrysanthemum is a highly heterozygous outcrossing hexaploid species with many chromosomes (2n = 6x = 54), which complicates genetic analyses. However, this problem is now being resolved using massive parallel sequencing technology. For example, van Geest et al. [17] showed that chrysanthemum’s inheritance mode is hexasomic, i.e. random chromosome pairing occurs during bivalent formation. Furthermore, quantitative trait loci have been detected using highly sophisticated methods that can handle genome complexity [16]. Moreover, Chrysanthemum seticuspe, a wild species of diploid chrysanthemum for which whole genome assembly has been performed previously, is established as a model strain in the genus Chrysanthemum [20, 33, 34]. Thus, the diploid C. seticuspe genome has been used as a reference for efficiently developing DNA markers in chrysanthemum cultivars [45]. Using the genome sequence of C. seticuspe as a reference for mining DNA polymorphisms from ddRAD-Seq data of hexaploid cultivated chrysanthemum, DNA markers linked to the resistance genetic locus Phr1 have been reported [46]. According to gene-for-gene interactions [14, 15], plants have evolved sophisticated resistance
systems against pathogens, whereas pathogens have evolved functions by which they evade these plant resistance systems. Through these interactions, plants have acquired many resistance genes. Based on this theory, chrysanthemum may have acquired multiple resistance genes against *P. horiana*. Previous inoculation experiments [7, 51] indicated that more useful resistance genes besides Phr1 likely exist in chrysanthemum cultivars. Identifying the molecular markers associated with other resistance genes will contribute to marker-assisted selection and promote systematic resistance breeding (e.g. pyramiding resistance genes).

This study aimed to develop a highly accurate DNA marker for a novel *P. horiana* resistance locus to aid marker-assisted selection and resistance detection. Previously, ddRAD-Seq analysis was used to develop chrysanthemum DNA markers [45, 46]. This approach is feasible for species with high heterozygosity and large genomes, such as chrysanthemum cultivars [13]. In ddRAD-Seq analysis, DNA polymorphisms are identified from restriction-site associated sequences. Thus, linkage between DNA markers and traits of interest is sometimes weak due to the genome complexity reduction. In addition, a single biparental mapping population might be insufficient to saturate DNA markers on a linkage map if genetic distance between the parents is close. Consequently, to design highly accurate DNA markers, obtaining information on sequences that exist in the gaps between ddRAD-Seq markers is necessary. Unfortunately, whole genome alignments of cultivated chrysanthemums are underdeveloped. Long-read sequencing technologies are currently the most powerful approach for obtaining unknown gap sequences; however, they are not cost-effective because the whole genome size of cultivated chrysanthemum is estimated to be as large as approximately 18 Gbp by flow cytometry [32]. Alternatively, short-read sequencing analysis can provide a large amount of data at a relatively low cost; thus, mapping such reads to the genome of a diploid wild species may offer a solution to this problem. Recently, a pseudomolecule of *C. seticuspe* was reported (http://mum-garden.kazusa.or.jp). Accordingly, using the genome sequence of *C. seticuspe* as a reference, we conducted whole genome sequencing and identified the sequence information needed to develop accurate DNA markers for chrysanthemum cultivars. We also tested the transferability and use of the resulting DNA markers in the genetic detection of *P. horiana* resistance.

**Results**

**Inheritance of *P. horiana* resistance in “Ariesu”**

The reaction of “Ariesu” and “Yellow Queen” to *P. horiana* isolates was investigated using the inoculation assay. “Ariesu” exhibited resistance to six isolates (TS, AK, IB, TO1, TO2 and TO3) but was susceptible to NA (Table 1; Fig. 1). “Yellow Queen” exhibited susceptibility to all isolates. Next, the reaction of the 283 F1 individuals originating from reciprocal crosses between “Ariesu” and “Yellow Queen”, respectively, at 35 days after inoculating the *P. horiana* isolate TS.

**GWAS and linkage analysis for *P. horiana* resistance in “Ariesu”**

Via GWAS, DNA markers associated with resistance in “Ariesu” were developed using biparental F1 populations. Approximately 2.1 M high-quality reads per sample were obtained. Of these sequence reads, 82.9% were mapped onto the *C. seticuspe* reference genome, CSE_r1.0 [20]. In total, 274700 SNP candidates were selected. After filtering with criteria of number of reads (≥10) and missing data (<0.75), 73025 high-confidence SNP candidates were identified. Of these, 1598 double-simplex (Aaaaaa × Aaaaaa or AAAAA × AAAAA) and 9181 simplex (Aaaaaa × aaaaa, aaaaa × Aaaaaa, AAAAAa × AAAAAA or AAAAA A × AAAAAA) SNPs were selected.

![“Ariesu” “Yellow Queen”](image)

Table 1. Reaction of “Ariesu”, “Yellow Queen” and the representative F1 plants to seven *P. horiana* isolates. Plants that showed no telia were scored as “R”. Plants were scored as “S” when at least one telium was observed on a plant. *P. horiana* resistance was examined by three separate inoculations per isolate.

| Cultivar and F1 individual | Reaction to *P. horiana* isolates |
|---------------------------|---------------------------------|
|                           | TS | NA | AK | TO1 | TO2 | TO3 |
| Ariesu                    | R  | S  | R  | R   | R   | R   |
| Yellow Queen              | S  | S  | S  | S   | S   | S   |
| YA-01                     | R  | S  | R  | R   | R   | R   |
| YA-03                     | R  | S  | R  | R   | R   | R   |
| YA-05                     | R  | S  | R  | R   | R   | R   |
| YA-07                     | R  | S  | R  | R   | R   | R   |
| YA-10                     | R  | S  | R  | R   | R   | R   |
| YA-14                     | R  | S  | R  | R   | R   | R   |
| YA-25                     | R  | S  | R  | R   | R   | R   |
| YA-52                     | R  | S  | R  | R   | R   | R   |
| YA-04                     | S  | S  | S  | S   | S   | S   |
| YA-09                     | S  | S  | S  | S   | S   | S   |
| YA-11                     | S  | S  | S  | S   | S   | S   |
| YA-26                     | S  | S  | S  | S   | S   | S   |
| YA-27                     | S  | S  | S  | S   | S   | S   |
| YA-29                     | S  | S  | S  | S   | S   | S   |
| YA-37                     | S  | S  | S  | S   | S   | S   |
| YA-43                     | S  | S  | S  | S   | S   | S   |

Figure 1. Abaxial sides of leaves of resistant “Ariesu” and susceptible “Yellow Queen”, respectively, at 35 days after inoculating the *P. horiana* isolate TS.
The generalised linear model analysis of 10,779 (i.e. 1,598 + 9,181) SNP markers identified 82 SNP markers that were significantly associated with \( P. \) horiana resistance (Supplementary data Table S4). The SNP marker SCSE_SC004884.1_65872 exhibited the highest association with the lowest p-value of 1.12 × 10\(^{-11}\). The ddRAD-Seq results showed that the genotypes of the SNP marker SCSE_SC004884.1_65872 in resistant “Ariesu” were TTTTTT, whereas those in susceptible “Yellow Queen” were TTTTGT. Additionally, the genotypes of SCSE_SC000716.1_75925, which had the second-lowest p-value of 2.76 × 10\(^{-16}\), were AAAAAG in “Ariesu” and AAAAAA in “Yellow Queen”. The minor C allele of SCSE_SC004884.1_65872 and the minor G allele of SCSE_SC000716.1_75925 were in the coupling phase of the resistant allele in Phr2 (Table 2).

Fine mapping by comparing “Ariesu” and “Yellow Queen” whole genome sequences

To obtain markers closely linked to the Phr2 locus, we analysed the sequences of genomic regions between the two markers SCSE_SC004884.1_65872 and SCSE_SC000716.1_75925 and designed primer pairs. Linkage analysis indicated that Phr2 was located at a genetic distance of 1.4 cm from SCSE_SC004884.1_65872 and 2.1 cm from SCSE_SC000716.1_75925 (Fig. 2A). The genome of cultivated chrysanthemums has not yet been elucidated; thus, the sequence between the two SNP markers is unclear. Therefore, we assumed that the pseudomolecule of diploid \( C. \) seticuspe could act as a reference genome for cultivated chrysanthemum. The base sequences associated with the SNP markers SCSE_SC004884.1_65872 and SCSE_SC000716.1_75925 were then positioned on chromosome 9 of the \( C. \) seticuspe pseudomolecule. This region spanned a physical interval of 7.4 Mb (Fig. 2B; SCSE_SC004884.1_65872 to SCSE_SC000716.1_75925: 44717622–52150792 bp).

SOAPdenovo2 was used to assemble 789,000 and 880,1 Gbp HiSeq paired-end reads of the whole “Ariesu” and “Yellow Queen” genomes (k-mer = 101), respectively. Overall, 101,290,730 and 97,821,561 contigs of “Ariesu” and “Yellow Queen”, respectively, were generated (Supplementary data Table S5), with the assembled genomes showing ultrafragmentation. The total and N50 lengths were 21,001,896,460 and 203 bp in “Ariesu” and 20,449,854,573 and 203 bp in “Yellow Queen”, respectively. We selected 3,743,825 and 3,521,557 contigs that were >500 bp in size of “Ariesu” and “Yellow Queen”, respectively (Supplementary data Table S5; accession numbers BOUO010000001–BOUO013743825 for “Ariesu” and BOUPO10000001–BOUPO13521557 for “Yellow Queen”), mapped these onto the \( C. \) seticuspe pseudomolecule and finally obtained the discontinuous genome sequences of “Ariesu” and “Yellow Queen” (Fig. 2B). Eight SNP markers were designed every 0.5 Mb in the 46.1–50.0 Mb region on chromosome 9 of the \( C. \) seticuspe pseudomolecule (Table 3).

These SNP markers were used to delimit the Phr2 locus with more precision. Of 656 \( F_1 \) plants, we identified 20 \( F_1 \) recombinant plants exhibiting genetic recombination between SCSE_SC004884.1_65872 and SCSE_SC000716.1_75925. Subsequently, we analysed the genotypes of these 20 plants with the 8 SNP markers. The Phr2 locus was narrowed to a 0.7-cM genetic interval between the SNP markers Cse2.0_LG9_46170750\(^{C} \), Cse2.0_LG9_46476925\(^{C} \) and Cse2.0_LG9_49032201A\(^{A} \) (Fig. 2C). The potential region between these markers in “Ariesu” should contain the gene conferring resistance and correspond to a region of 2.6 Mb (Cse2.0_LG9_46476925\(^{C} \) to Cse2.0_LG9_49032201A\(^{A} \): 46476925–49032201 bp) on chromosome 9 of the \( C. \) seticuspe pseudomolecule.

Markers for detecting \( P. \) horiana resistance

Four SNP markers (Cse2.0_LG9_47009004, Cse2.0_LG9_47483611, Cse2.0_LG9_48084985 and Cse2.0_LG9_48453417) co-segregated with Phr2 in the 656 \( F_1 \) progenies (Fig. 2C), suggesting that they were in linkage disequilibrium with Phr2 and that cultivars with Phr2 resistance-conferring genes could be identified among the chrysanthemum genetic resources. To assess transferability, we tested associations with resistance to \( P. \) horiana isolates in the 46 cultivars (Table 4). None of the 7 isolates showed an identical infection profile on the set of 46 cultivars, indicating that each of them represents a different pathotype. TS infected the largest number of cultivars (25 out of 46), NA and TO2 also infected many cultivars, 22 and 21 cultivars, respectively. TO3 infected the smallest number of cultivars (13 out of 46). Seven isolates showed non-redundant infection interaction with at least three cultivars. A set of “Purinesusu”, “SEI17” and “Southern Shell” or a set of “Purinesusu”, “SEI17” and “Refour” can be used as differential cultivars to distinguish the seven isolates.

Phr2, developed from “Ariesu”, conferred multiple isolate-specific resistance against six isolates, TS, AK, IB, TO1, TO2 and TO3, with susceptibility to NA, as described in the section Inheritance of \( P. \) horiana resistance in “Ariesu”. Three cultivars, namely “Kyura Shusa”, “SEI01” and “SEI02”, showed the same interaction phenotype against isolates, and each possessed all the resistance alleles for the four SNPs associated with Phr2, strongly indicating that these cultivars carry Phr2. Ten other cultivars, “Kyura Kids”, “Moze Cute”, “SEI03”, “SEI04”, “SEI05”, “SEI06”, “SEI07”, “SEI08”, “Southern Pegasus” and “TM Miruku” showed resistance to all the tested isolates. Two of these, “Moze Cute” and “SEI03”, had all the resistance alleles for the four SNPs, indicating that Phr2 confers resistance in these two cultivars. The interaction phenotype of complete resistance to the seven isolates indicated that “Moze Cute” and “SEI03” likely carry other resistance genes or alleles that confer resistance to the isolate NA because only Phr2 was susceptible to NA. Ten cultivars from “Kanran” to “Snowdon” listed in Table 4 as well as “Yellow Queen” showed susceptibility to all the seven isolates.

We also investigated marker–resistance association in the progenies of five resistant cultivars carrying the resistance alleles for SNPs associated with Phr2. “Kyura Shusa”, “Moze Cute”, “SEI01”, “SEI02” and “SEI03”. \( F_1 \) populations were obtained from crosses with the susceptible “Yellow Queen” and subjected to inoculation tests using the TS isolate and PCR analysis to identify the representative Cse2.0_LG9_48084985\(^{C} \) SNP marker. The close to 1:1 segregation of resistance and susceptibility in \( F_1 \) populations derived from “Kyura Shusa”, “SEI01” and “SEI02”, along with resistance against the TS isolate, was consistent with the presence of the resistant C allele at the SNP marker Cse2.0_LG9_48084985\(^{C} \) (Table 5). The results demonstrated the marker–resistance association in these cultivars and confirmed that the SNP marker could be used for detecting resistance. Based on the proportion of resistant plants in the \( F_1 \) populations obtained from the male parent “Moze Cute” and “SEI03”, it was concluded that these cultivars have multiple genetic loci conferring resistance against \( P. \) horiana. In the \( F_1 \) population derived from “Moze Cute”, the resistance-susceptibility ratio was 35:9 (Table 5). A segregation ratio of 4:1 was observed in the two genetic nulliplex (AAaaaa × aaaaaa) or two genetic loci of simplex × nulliplex on different homologous chromosome groups (AAAAaa × aaaaaa and BBBBbb × bbbbbb). Furthermore, this segregation ratio fits well into the 4:1 ratio \( (\chi^2 = 0.0057, p = 0.94)\) of the duplex × nulliplex compared with the 3:1 ratio \( (\chi^2 = 0.48, p = 0.47)\) of the two genetic loci of simplex × nulliplex; however, the population size was not...
Table 2. Relationship between marker genotypes and \textit{P. horiana} resistance in 283 \textit{F1} plants obtained from reciprocal crosses between “Ariesu” and “Yellow Queen”

| Marker genotype\(^a\) | \textit{P. horiana} resistance\(^b\) | Resistant | Susceptible |
|------------------------|---------------------------------|-----------|-------------|
| TTTTTC                 | AAAAG                           | 142       | 0           |
| TTTTTC                 | AAAAAA                          | 0         | 1           |
| TTTTTT                 | AAAAG                           | 3         | 6           |
| TTTTTT                 | AAAAAA                          | 0         | 131         |

\(^a\)The genotypes of SCSE\_SC004884\_1\_65872 in resistant “Ariesu” and susceptible “Yellow Queen” were TTTTTC and TTTTTT, respectively. The genotypes of SCSE\_SC000716\_1\_75925 in resistant “Ariesu” and susceptible “Yellow Queen” were AAAAAAG and AAAAAAA, respectively. \(^b\)Plants that showed no telia were scored as resistant. Plants were scored as susceptible when at least one telium was observed. \textit{P. horiana} resistance was examined by three separate inoculations using the isolate TS.

hexaploid chrysanthemum “Ariesu” and “Yellow Queen”

\[ \text{F}_1 \text{ population} \rightarrow \text{whole genome shotgun sequencing} \rightarrow \text{ddRAD-Seq and GWAS} \rightarrow \text{SNP markers} \rightarrow \text{alignment onto} \ C. \text{seticuspe pseudomolecule} \]

Figure 2. Overview of the process for developing single nucleotide polymorphism (SNP) markers and fine mapping for \textit{Phr2} locus with a combination of genome-wide association study and whole genome sequencing in chrysanthemum cultivars using the genome of a wild species, diploid \textit{C. seticuspe}, as a reference. (A) Partial linkage map containing the \textit{Phr2} locus. The SNP markers SCSE\_SC004884\_1\_65872 and SCSE\_SC000716\_1\_75925 were obtained by ddRAD-Seq analysis and association study in the \textit{F1} biparental population derived from reciprocal crosses between “Ariesu” and “Yellow Queen” (\(n=283\)). Genetic distances (cM) are indicated on the left. (B) Assembled genome sequences of “Ariesu” and “Yellow Queen” (short grey lines) mapped onto the \textit{C. seticuspe} chromosome (bold line) for identifying SNP markers between ddRAD-Seq markers. The corresponding positions of the markers were determined on the \textit{C. seticuspe} genome sequence of chromosome 9 (CSE\_r2.0, http://mum-garden.kazusa.or.jp). Positions (bp) are indicated on the left. (C) Fine map of \textit{Phr2} generated from the \textit{F1} biparental population derived from reciprocal crosses between “Ariesu” and “Yellow Queen” (\(n=656\)) using SNP markers obtained by comparing the assembled genome sequences of parents. Genetic distances (cM) and marker names are indicated on the left and right, respectively.
sufficiently large to draw a definitive conclusion. Nevertheless, from these data, it can be estimated that "Moze Cute" has at least two resistance loci. In this population, the resistant C allele at the SNP marker Cse2.0_LG9_48084985 was segregated in a ratio of 23:21 (i.e. almost 1:1), and every plant carrying the allele was resistant to the TS isolate. Thus, "Moze Cute" apparently had a resistance against the TS isolate. Similarly, in the F1 population obtained from "Sanyo-ougon" and "Seiko-no-mine", the ratio of almost 4:1, suggesting a duplex. Additionally, five F1 plants with no resistance allele exhibited resistance, indicating that at least two of those were Phr2 because the Phr2-resistant allele was segregated in the ratio of almost 4:1, suggesting a duplex. Additionally, five F1 plants with no resistance allele exhibited resistance, indicating that "SEI03" carried another resistance gene or allele. Consequently, the results for "Moze Cute" and "SEI03" validated the SNP marker Cse2.0_LG9_48084985 as a tool for distinguishing Phr2 resistance in these plants. Similarly, in the F1 population obtained from "SEI03", the segregation ratio of 42:6 indicated the presence of multiple resistance genes or alleles in "SEI03". At least two of those were Phr2 because the Phr2-resistant allele was segregated in the ratio of almost 4:1, suggesting a duplex. Additionally, five F1 plants with no resistance allele exhibited resistance, indicating that "SEI03" carried another resistance gene or allele. Consequently, the results for "Moze Cute" and "SEI03" validated the SNP marker Cse2.0_LG9_48084985 as a tool for distinguishing Phr2 resistance in these plants.

Discussion

Three types of P. horiana resistance have been reported in chrysanthemum: complete resistance (no spore production), incomplete resistance (limited spore production) and necrosis (necrotic areas develop around the growing rust colonies and sporulation may not be completely inhibited) [8]. In this study, complete resistance was determined as "R" using a simple scoring system, in which the responses of incomplete resistance, necrosis and susceptibility were scored as "S". Multiple cultivars with complete resistance were identified. Plants have evolved sophisticated resistance systems against pathogens and acquired many resistance genes in a gene-for-gene manner [14, 15]. In wheat, 79 resistance loci have been identified [19, 37, 39, 43]. In barley, 26 resistance loci were identified [24,36,39]. In maize, 20 genes conferring resistance to Puccinia sorghi were previously identified [18, 22, 41, 50]. Similarly, our results suggested that multiple resistance genes against P. horiana exist in chrysanthemum genetic resources [7, 8, 31, 51]. Specifically, we identified a group of resistance cultivars carrying Phr2. Furthermore, we found three other groups of cultivars that showed the same interaction phenotype profile against seven isolates (Table 4): (1) a group including "Kankohbai", "SEI09" and "SEI10", (2) a group including "Sanyo-ougon", "Seiko-no-mine", "Westland Pink", "SEI14", "SEI15" and "SEI16" and (3) a group with "Kanseisetsu" and "Otomozakura". Cultivars in each group likely carry the same type of resistance gene. A non-redundant differential interaction phenotype was also observed in 10 cultivars: "Moze Frame", "Kanamarufuji", "Purinsesu", "Bunmei", "SEI17", "Southern Shell", "Refour", "Seikonohikari", "Jimba", and "Meimon". These cultivars had none of the resistance alleles for Phr2; therefore, it was inferred that they carry different resistance genes.

In the commercial cultivation of chrysanthemum, it is desirable to completely eliminate or control white rust. Compared with incomplete resistance and necrosis, complete resistance has the advantage of the complete absence of P. horiana. In addition, complete resistance shows dominant inheritance, i.e. simple Mendelian inheritance is shown even in hexaploid chrysanthemums [8, 46, 51]. In contrast, the probability of progenies inheriting incomplete resistance or necrosis is generally low [8, 51]. Thus, identifying complete resistance is advantageous for breeding resistant cultivars and achieving marker-assisted selection. Based on the present results, identifying Phr2 resistance-conferring genes among the available chrysanthemum genetic resources is possible. So far, Phr1 has been reported as a complete resistance locus. However, our findings (Table 4) suggest that there are other genes conferring complete resistance in chrysanthemum genetic resources. Resistance detection is also expected to become possible when a highly accurate DNA marker set is provided for analysing other genes conferring complete resistance against P. horiana.

Genetic analysis, DNA marker development and whole genome assembly have been difficult in chrysanthemum cultivars due to their high heterogeneity, hexaploidy and large genome size. In this study, highly accurate SNP markers were developed by fine mapping following GWAS. Previous studies have also described marker development via GWAS based on ddRAD-Seq in biparental F1 populations of chrysanthemum cultivars [45, 46]. In this study, highly accurate SNP markers were developed by fine mapping following GWAS. Previous studies have also described marker development via GWAS based on ddRAD-Seq in biparental F1 populations of chrysanthemum cultivars [45, 46]. In contrast, the N50 lengths were relatively short, i.e. they were fragmentarily. Such fragmentation was similarly observed in the de novo assemblies of diploid Chrysanthemum species genomes obtained at an early stage [20, 44] because repetitive sequences occupied >68.6% of the whole genome in the genus Chrysanthemum [20, 29, 44, 49]. In this study, the high ratio of repetitive sequences could be a major cause of the failure in assembling longer sequences in cultivated chrysanthemums. We mapped these assembled genome sequences onto the C. seticuspe pseudomolecule and were able to identify base variants. Indeed, we treated the C. seticuspe pseudomolecule as the de facto monoploid genome of hexaploid chrysanthemum cultivars. The resulting SNP markers were validated by fine

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**Table 3. Single nucleotide polymorphisms (SNPs) identified between SCSE_SC004884.1_65872 and SCSE_SC000716.1_75925**

| SNPs | Parental genotype | Corresponding SNP position on chromosome 9 of the C. seticuspe pseudomolecule (bp) |
|------|-------------------|----------------------------------------------------------------------------------|
| Cse2 0.1_LG9_46170750 | CCCCCCT | 46170750 |
| Cse2 0.1_LG9_46476925 | TTTTTC | 46476925 |
| Cse2 0.1_LG9_47009004 | AAAAGA | 47009004 |
| Cse2 0.1_LG9_47483611 | GGGGTT | 47483611 |
| Cse2 0.1_LG9_48084985 | TTTTTC | 48084985 |
| Cse2 0.1_LG9_48453417 | GGGGGA | 48453417 |
| Cse2 0.1_LG9_49032201 | GGGGGA | 49032201 |
| Cse2 0.1_LG9_50042598 | GGGGGA | 50042598 |

SNPs were designed every 0.5 Mbp in the 46.1–50.0-Mbp region on the C. seticuspe pseudomolecule.
Table 4. Resistance to seven *P. horiana* isolates and presence of resistance allele in Phr2-linked single nucleotide polymorphism (SNP) markers in chrysanthemum genetic resources. Cultivars that showed no telia were scored as “R”. Cultivars were scored as “S” when at least one telium was observed on the plant. *P. horiana* resistance was examined by three separate inoculations per isolate.

| Cultivar         | Resistance to *P. horiana* isolates | Presence of resistance allele in Phr2-linked SNP markers<sup>a</sup> |
|------------------|-------------------------------------|---------------------------------------------------------------|
|                  | TS  | NA  | AK  | IB  | TO1 | TO2 | TO3 | Cse2.0_LG9_47009004<sup>G</sup> | Cse2.0_LG9_47483611<sup>T</sup> | Cse2.0_LG9_48084985<sup>C</sup> | Cse2.0_LG9_48453417<sup>^A</sup> |
| Ariesu           | R   | S   | R   | R   | R   | R   | R   | +                          | +                          | +                          | +                          |
| Kyura Kids       | R   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| Moze Cute        | R   | R   | R   | R   | R   | R   | R   | +                          | +                          | +                          | +                          |
| SEI03            | R   | R   | R   | R   | R   | R   | R   | +                          | +                          | +                          | +                          |
| SEI04            | R   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| SEI05            | R   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| SEI06            | R   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| SEI07            | R   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| SEI08            | R   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| Southern Pegasus | R   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| TM Minuku        | R   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| Kyura Shusa      | R   | S   | R   | R   | R   | R   | R   | +                          | +                          | +                          | +                          |
| SEI01            | R   | S   | R   | R   | R   | R   | R   | +                          | +                          | +                          | +                          |
| SEI02            | R   | S   | R   | R   | R   | R   | R   | +                          | +                          | +                          | +                          |
| Kankohbai        | R   | R   | R   | R   | R   | R   | S   | −                          | −                          | −                          | −                          |
| SEI09            | R   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| SEI10            | R   | R   | R   | R   | R   | R   | S   | −                          | −                          | −                          | −                          |
| Sanyo-ougon      | S   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| Seiko-no-mine    | S   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| Westland Pink    | S   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| SEI14            | S   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| SEI15            | S   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| SEI16            | S   | R   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| Kaneisetsu       | S   | R   | S   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| Otomezakura      | S   | R   | S   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| Moze Frame       | R   | S   | R   | R   | R   | R   | S   | −                          | −                          | −                          | −                          |
| Kanamaruji       | S   | S   | R   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| Purinesu         | R   | S   | R   | R   | S   | S   | R   | −                          | −                          | −                          | −                          |
| Bunmei           | S   | R   | S   | R   | S   | R   | R   | −                          | −                          | −                          | −                          |
| SEI17            | S   | S   | S   | R   | R   | R   | R   | −                          | −                          | −                          | −                          |
| Southern Shell   | R   | R   | S   | R   | S   | S   | S   | −                          | −                          | −                          | −                          |
| Refour           | R   | R   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| Seikohokari      | S   | S   | R   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| Jimba            | S   | R   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| Meimon           | S   | S   | S   | S   | S   | S   | R   | −                          | −                          | −                          | −                          |
| Kanran           | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| Kinkou           | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| Penny Lane       | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| SEI11            | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| SEI12            | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| SEI13            | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| SEI18            | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| Shuho-no-chikara | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| Shuho-no-kokoro  | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| Snowdon          | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |
| Yellow Queen     | S   | S   | S   | S   | S   | S   | S   | −                          | −                          | −                          | −                          |

<sup>a</sup>These SNP markers co-segregated with Phr2 in 656 F<sub>1</sub> progenies derived from reciprocal crosses between "Ariesu" and "Yellow Queen". +, presence; −, absence.

Mapping and showed transferability; therefore, the assembled genome sequences of the chrysanthemum cultivars were reliable.

The genetic order of SNP markers in the chrysanthemum linkage map was consistent with their physical positions in the *C. seticuspe* pseudomolecule, suggesting that chrysanthemum cultivars have collinearity in this genomic region with *C. seticuspe*. Collinearity between the genomes of polyploid crops and their diploid relatives has previously been reported in strawberry [10], sugarcane [48] and sweet potato [42]. Generally, robust resistance genes of cultivars are derived from ancestral donors of wild species [12, 47, 52]. A homologous gene of Phr2 may be found in the *C. seticuspe* genome given that *C. seticuspe* is also resistant to *P. horiana* [Yamaguchi [51], who classified *C. seticuspe* as *C. boreale*]. Recently, a chromosome-level genome sequence of *C. seticuspe* was elucidated [33]. Thus, we searched the *C. seticuspe* genome for candidate genes in a 2.6-Mb genome region on chromosome 9, corresponding to a Phr2 candidate locus in "Ariesu". In total, 4 genes related to disease resistance (CsG_LG9.jg218308.t1.g000908.1, CsG_LG9.jg218318.t1.g000918.1, CsG_LG9.jg218352.t1.g000952.1 and CsG_LG9.jg218356.t1.g000956.1) were identified from 30
predicted genes found in the examined *C. seticuspe* region. Each gene encoded immune receptors of the nucleotide-binding site leucine-rich repeat (NBS-LRR) protein; this gene class is involved in disease resistance in plants [11]. However, we could not search for a *Phr2* locus in “Ariesu” with such NBS-LRR genes because the genome sequence is fragmented and discontinuous. In future studies, the collinearity may be used for the positional cloning of causal genes in chrysanthemum cultivars.

In conclusion, this study identified a new resistance locus and DNA markers for resistance against white rust caused by *P. horiana* in chrysanthemum cultivars using ddRAD-Seq analysis and assembled genome sequence comparison with wild *C. seticuspe* pseudomolecule as a *de facto* monoploid genome. Notably, although the assembled genome sequence of the chrysanthemum cultivar was severely fragmented, it could be reconstructed with satisfactory quality for DNA marker development by mapping onto the *C. seticuspe* pseudomolecule. Our results demonstrated this method’s utility in providing a model for DNA marker development in chrysanthemum cultivars.

### Materials and methods

#### Plant materials

We prepared 46 cultivars, which were selected based on previous reports [8, 23, 31, 38, 46, 51] and anecdotal reports of infections from breeders. The details of these cultivars are provided in Supplementary data Table S1.

A pair of *F*₁ populations originating from reciprocal crosses between spray-type “Ariesu” and spray-type “Yellow Queen” was produced. In total, 64 seedlings from “Ariesu” × “Yellow Queen” and 219 seedlings from “Yellow Queen” × “Ariesu” were obtained for ddRAD-Seq and genome-wide association study (GWAS).

Another *F*₁ population (*n* = 373) additionally obtained from “Yellow Queen” × “Ariesu” was used for fine-mapping analysis. Using several resistance spray-type cultivars (“Kyura Shusa”, “Moze Cute”, “SEI01”, “SEI02” and “SEI03”) as pollen parents, crosses with “Yellow Queen” were made, resulting in 54, 44, 36, 50 and 48 *F*₂ seedlings, respectively, for the analysis of single nucleotide polymorphism (SNP) marker–resistance association.

Stock plants were planted in plastic pots (internal diameter, 12 cm; one stock plant for each genotype per pot) containing horticultural soil (Yokabaido, Hokkaido Peatmoss Co., Ltd.) and maintained in the vegetative state under 6-h night-break conditions (natural photoperiod with a night-break from 2200 to 0400 h using fluorescent white-light lamps) in a glasshouse at 18°C–25°C. Cuttings for the inoculation assay and samples for DNA extraction were obtained from the stock plants.

#### Fungal isolates

Seven *P. horiana* isolates (codes: TS, NA, AK, IB, TO1, TO2 and TO3) were collected from cut flower growers or agricultural experimental stations in Japan in 2018 (Supplementary data Table S2). *P. horiana* is an autoecious fungus; thus, each isolate was maintained on *P. horiana*-free plants (susceptible “Shuho-no-chikara”) as described by Alaei et al. [1] and De Backer et al. [7]. The plants were grown in growth chambers maintained at 20°C and 70% relative humidity (RH) with a 16-h photoperiod provided using fluorescent white-light tubes.

#### Inoculation method

Inoculation was performed in a polystyrene foam box as described by Sumitomo et al. [46]. Briefly, chrysanthemum rooted cuttings were put at the bottom of the box. The top opening of the box was covered with a 5-mm mesh plastic net. The heavily infected leaves were placed on the net with the telia pointing downwards. Demineralised water was used to mist the cuttings, inoculum and inside of the box. After the box was closed, it was placed in a growth chamber under dark conditions at 19°C. Subsequently, 16 h after inoculation, the rooted cuttings were transferred into a growth chamber (22°C, 70% RH, 16-h photoperiod). Symptoms were evaluated 35 days after inoculation.

Inoculation assays were performed to investigate the susceptibility of the 46 cultivars to each of the 7 *P. horiana* isolates (TS, NA, AK, IB, TO1, TO2 and TO3). Isolate TS was used for *F*₂ populations originating from crosses between “Yellow Queen” and cultivars “Ariesu”, “Kyura Shusa”, “Moze Cute”, “SEI01”, “SEI02” and “SEI03”. One rooted cutting per genotype was used in one assay, and the assay was repeated three times per isolate in different experiments.

#### Phenotyping

A simple scoring system, susceptible (S) or resistant (R), was used to identify complete resistance, which is the strongest of the three *P. horiana* resistance types [8]. If at least one telium was observed on a plant in any of the assays, the phenotype was scored as “S”, whereas plants that showed no telia at all were scored as “R”.

### Table 5. Relationship between the genotype of *Cse2.0_LG9_48084985C* and resistance to the *P. horiana* isolate TS

| Resistant parent | Resistant C allele of *Cse2.0_LG9_48084985C* | Resistance to the *P. horiana* isolate TS |
|------------------|---------------------------------------------|------------------------------------------|
| Kyura Shusa      | Presence                                    | 25                                       |
|                  | Absence                                     | 0                                        |
| SEI01            | Presence                                    | 17                                       |
|                  | Absence                                     | 0                                        |
| SEI02            | Presence                                    | 23                                       |
|                  | Absence                                     | 0                                        |
| Moze Cute        | Presence                                    | 23                                       |
|                  | Absence                                     | 0                                        |
| SEI03            | Presence                                    | 37                                       |
|                  | Absence                                     | 5                                        |
ddRAD-Seq analysis

F1 populations originating from “Ariesu” × “Yellow Queen” (n = 64) and “Yellow Queen” × “Ariesu” (n = 219) and the parents (“Ariesu” and “Yellow Queen”) were used. Genomic DNA was isolated from the shoot tips (fresh weight, 30 mg) using a DNaseasy Plant Mini Kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). ddRAD-Seq libraries were prepared as described by Sumitomo et al. [45] and sequenced using HiSeq 2000 and MiSeq platforms (Illumina). The sequence reads were registered in the Sequence Read Archive database in the DNA Data Bank of Japan (accession numbers DRA007925, DRA011746 and DRA011899).

Data processing and simplex SNP mining

Data processing of sequence reads and simplex SNP calling were performed as described by Sumitomo et al. [45]. Briefly, high-quality ddRAD-Seq reads were mapped onto the reference C. seticuspe genome sequence (CSE_r1.0; [20]) using Bowtie 2 (version 2.2.3; [26]) with parameters of maximum fragment size length, 1000 (I = 1000), in the “—sensitive” preset of the “—end-to-end” mode. The resultant sequence alignment/map format files were converted to binary sequence alignment/map format files and subjected to SNP calling using the mpileup option of SAMtools (version 0.1.19; [28]) and the mpileup2snp option of VarScan 2 (version 2.3; [25]) to obtain a variant call format file that included SNP information. High-confidence SNPs were called from the resulting sequence alignments using the following criteria in VCFtools (version 0.1.12b; [6]): (i) depth of coverage: ≥10 for each SNP position in each F1 individual; (ii) proportion of F1 individuals with missing data in F1 population: <0.25 for each locus.

The number of reads in the pooled F1 progeny samples was used to estimate the genotype of the parental cultivars at each SNP locus [2, 42]. Simplex and double-simplex SNPs were selected according to the alternative allele frequency (AAF) of the pooled SNP locus [2, 42]. Simplex and double-simplex SNPs were selected to estimate the genotype of the parental cultivars at each SNP position in each F1 individual; segregation of the minor G allele on SCSE_SC000716.1_75925 were screened by polymerase chain reaction (PCR) analysis using DNA from “Ariesu”, “Yellow Queen” and “Yellow Queen”, respectively, as SNP marker candidates.

GWAS

For a broadly diverse population, typical GWAS is performed. However, we used biparental populations for GWAS, wherein the SNP allele frequency could be predicted. The association study was performed in F1 populations consisting 64 seedlings from “Ariesu” × “Yellow Queen” and 219 seedlings from “Yellow Queen” × “Ariesu” using the TASSEL programme (with the default parameters) to run a general linear model [5]. Thresholds for the association were set at 4.6 × 10−6 (= 0.05/10779) at a 5% significance level after implementing a Bonferroni multiple test correction [4].

Linkage analysis

Linkage analysis was performed using JoinMap v4.1 software (Kyazma B.V., Wageningen, The Netherlands) based on the backcross (BC1) option with a limit of determination threshold of 10.0. The genetic distance between markers in a map was calculated using regression mapping and the Kosambi mapping function.

Fine mapping by comparing “Ariesu” and “Yellow Queen” whole genome sequences

The genomic DNA of “Ariesu” and “Yellow Queen” was used to construct paired-end libraries. The expected insert size of the paired-end libraries was 500 bp, and the sequences were generated using Illumina HiSeqX (Illumina, San Diego, CA, USA) with a 151-nt read length. The generated reads were assembled using SOAPdenovo2 with a k-mer of 101 [30]. Assembled sequences that were >500 bp in size were mapped onto the C. seticuspe pseudo-molecule (CSE_r2.0; http://mum-garden.kazusa.or.jp) using minimap2 software [27].

We used IGV software [40] to browse and compare the resultant sequence alignments and identified base variants between ddRAD-Seq markers, SCSE_SC004884.1_65872 and SCSE_SC000716.1_75925. The corresponding positions of these markers were 44,717,622 and 52,150,792 bp, respectively, on the C. seticuspe chromosome 9. Therefore, we identified SNPs from the assembled genome sequences of “Ariesu” and “Yellow Queen” mapped onto the 44,717,622 – 52,150,792-bp region of the C. seticuspe chromosome 9. SNPs determined as heterozygous and homozygous were identified in “Ariesu” and “Yellow Queen”, respectively, as SNP marker candidates.

Analysis of SNP marker–resistance association

SNP marker–resistance association was investigated in the genetic resources. For 46 cultivars, the presence or absence of the resistance-linked allele was investigated at four SNP positions (Cse2.0_LG9_47009004G, Cse2.0_LG9_47483611T, Cse2.0_LG9_48084985C and Cse2.0_LG9_48453417A) co-segregated with the minor C allele on CSE_SC004884.1_65872 and minor G allele on SCSE_SC000716.1_75925 were screened by polymerase chain reaction (PCR) analysis using DNA from “Ariesu”, “Yellow Queen” and the parents (“Ariesu” × “Yellow Queen”) were used. Genomic DNA was isolated from the shoot tips (fresh weight, 30 mg) using a DNeasy Plant Mini Kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany).

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**Author contributions**

KSu designed and executed the study, prepared all tables and figures and wrote the manuscript. KSh assisted in ddRAD-Seq analysis and GWAS. HH, KSu and SI conducted comparative genome analysis. AH conducted part of the inoculation test. MN, YN, TH, MK, HY and FT contributed to data analysis and redrafting of the manuscript.

**Availability of data and materials**

All phenotypic and genotypic data are provided as Supplementary Material.

**Conflicts of interest**

The authors declare that they have no competing interests.

**Supplementary data**

Supplementary data is available at Horticulture Research online.

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