

Full Paper

# Development of molecular markers associated with resistance to *Meloidogyne incognita* by performing quantitative trait locus analysis and genome-wide association study in sweetpotato

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## Abstract

The southern root-knot nematode, *Meloidogyne incognita*, is a pest that decreases yield and the quality of sweetpotato [*Ipomoea batatas* (L.) Lam.]. There is a demand to produce resistant cultivars and develop DNA markers to select this trait. However, sweetpotato is hexaploid, highly heterozygous, and has an enormous genome (~3 Gb), which makes genetic linkage analysis difficult. In this study, a high-density linkage map was constructed based on retrotransposon insertion polymorphism, simple sequence repeat, and single nucleotide polymorphism markers. The markers were developed using F<sub>1</sub> progeny between J-Red, which exhibits resistance to multiple races of *M. incognita*, and Choshu, which is susceptible to multiple races of such pest. Quantitative trait locus (QTL) analysis and a genome-wide association study detected highly effective QTLs for resistance against three races, namely, SP1, SP4, and SP6-1, in the Ib01-6 J-Red linkage group. A polymerase chain reaction marker that can identify genotypes based on single nucleotide polymorphisms located in this QTL region can discriminate resistance from susceptibility in the F<sub>1</sub> progeny at a rate of 70%. Thus, this marker could be helpful in selecting sweetpotato cultivars that are resistant to multiple races of *M. incognita*.

**Key words:** sweetpotato, GWAS, QTL mapping, polyploids, marker-assisted breeding

## 1. Introduction

Research in genomics and genetics has accelerated molecular breeding strategies for several crop species. In particular, high-density

genetic linkage maps are crucial tools for gene identification, quantitative trait locus (QTL) survey, comparative genomic analyses, and marker-assisted breeding programs. The polyploid nature of several

crop species makes genetic linkage analysis difficult. Polyploidy exists in crops, such as potato (*Solanum tuberosum*; tetraploid, 4x), canola (*Brassica napus*; tetraploid, 4x), bread wheat (*Triticum aestivum*; hexaploid, 6x), sweetpotato [*Ipomoea batatas* (L.) Lam.; hexaploidy, 6x], strawberry (*Fragaria x ananassa*; octaploid, 8x), and sugarcane (*Saccharum* spp.; 10x–12x). In general, genomic analyses in polyploid species can be performed by comparing the polyploid genome with a closely related diploid (2x) species.<sup>1–4</sup> However, genetic linkage analyses of polyploid species are more complicated than those of diploids because more variations in allele dosage is apparent in polyploids. During linkage analyses, the marker dosage is based on the segregation ratios of the mapping population. Thus, we need to consider numerous variations of the segregation ratios in polyploids. Those variations can also be altered by cytological characteristics, such as auto or allopolyploidy. Therefore, genetic linkage analyses in polyploid species are still challenging, although there are several excellent papers achieving map-based cloning on the hexaploid wheat.<sup>5–9</sup>

Sweetpotato is one of the most important crop species worldwide, and its global production exceeds 100 million tonnes.<sup>10</sup> This crop contains valuable nutrients, such as vitamins and minerals, and is widely cultivated in tropical and temperate regions. It is used in a wide range of goods, including processed foods, feeds, pigment materials, and ingredients of alcohol products. Thus, a growing demand for this crop indicates that the development of cultivars with disease and pest resistance is required to avoid commercial yield loss. However, genetic linkage analysis and marker-assisted breeding in sweetpotato have been challenging because of the complexity of its genomic architecture that comprises hexaploidy ( $2n = 6x = 90$ ), high heterozygosity, a huge genome (approximately 2–3 Gb), and outcrossing nature.

One of the important target traits in sweetpotato breeding is resistance to the southern root-knot nematode (*Meloidogyne incognita*), which is a major pest that decreases yield and the quality of sweetpotato.<sup>11</sup> The nematode invades roots, which results in the generation of spindle-shaped swellings (galls) on the fibrous roots and cracks on tuberous roots. In Japan, *M. incognita* can be found in most areas, including Kyushu and Kanto, in which sweetpotato is cultivated.<sup>12–14</sup> Previous studies have reported that at least nine races of *M. incognita* (SP1–SP9) exist in Japan, and certain races are dominant in specific regions.<sup>15</sup> For example, the SP1 race is predominant in the Kumamoto, Nagasaki, and Saga Prefectures (Kyushu); in contrast, the SP4 and SP6 races are predominant in the Okinawa islands<sup>15</sup> and are found in the Ibaraki and Chiba Prefectures (Kanto)<sup>16,17</sup>. Recently, Tabuchi et al.<sup>18</sup> have reported that SP6 could be classified into two races (SP6-1 and SP6-2) and SP6-1 was identified in Chiba. Thus, the development of cultivars resistant to the multiple races of *M. incognita* is highly desirable.

To date, several studies have reported about the construction of linkage maps in sweetpotato using amplified fragment length polymorphism (AFLP), sequence-related amplified polymorphism, simple sequence repeat (SSR), and retrotransposon-based markers.<sup>19–26</sup> Moreover, Shirasawa et al.<sup>27</sup> have established high-density single nucleotide polymorphism (SNP) linkage maps in sweetpotato utilizing the next-generation sequencing (NGS) platform. They performed double-digest restriction site-associated DNA sequencing (ddRAD-seq) analysis in an  $S_1$  mapping population derived from the self-pollination of sweetpotato cultivar Xushu18, which produced 28,087 SNP markers mapped onto 96 linkage groups (LGs) covering a total distance of 33,020.4 cM. This study first constructed a high-density linkage map containing >10,000 markers in sweetpotato using NGS technology.

In the present study, we performed linkage analysis to identify genomic regions controlling *M. incognita* resistance in sweetpotato. Our study focussed on the following three areas: (i) high-density

linkage map construction using several kinds of molecular markers, including SSRs, retrotransposons, and SNPs, via ddRAD-seq analysis in  $F_1$  mapping populations derived from resistant and susceptible cultivars; (ii) identification of the genomic regions that control resistance to the several races of *M. incognita* (SP1, SP4, and SP6-1) by QTL mapping and genome-wide association study (GWAS); and (iii) development of practical polymerase chain reaction (PCR)-based DNA markers for screening *M. incognita*-resistant plants.

## 2. Materials and methods

### 2.1. Plant materials

An  $F_1$  mapping population comprising 113 progeny lines was constructed by crossing J-Red with Choshu cultivars. J-Red is an orange-fleshed sweetpotato cultivar, which contains high levels of  $\beta$ -carotene and is highly resistant to most races of *M. incognita*, except SP8.<sup>15,18,28</sup> In contrast, Choshu is a local cultivar that is susceptible to the *M. incognita* races, SP1, SP2, SP3, SP4 SP6-1, and SP6-2.<sup>18</sup> A total of 107 of 113  $F_1$  progeny lines and two parental cultivars were used for *M. incognita* resistance testing and genetic analysis, because the remaining 6 lines withered after DNA extraction. The total DNA of the  $F_1$  lines and the parental cultivars was extracted from the leaves of plants using the DNeasy Plant Mini Kit (Qiagen Inc., Germany). DNA quantification and quality checks were performed using an ND1000 NanoDrop spectrophotometer (NanoDrop Technologies, DE, USA).

### 2.2. Marker development

Several types of DNA markers, including retrotransposon-based, SSR, and SNP markers, were developed to construct the linkage maps. To develop retrotransposon-based molecular markers, insertion sites for the active retrotransposon families, namely, *Rtsp-1*<sup>29</sup> and *Cl8*, were sequenced using the Illumina MiSeq sequencing system. *Cl8* was a novel retrotransposon family, which was identified using the method developed by Monden et al.<sup>30</sup> We experimentally confirmed that these families exhibit high insertion polymorphisms among sweetpotato cultivars and achieved relatively high copy numbers in the sweetpotato genome.<sup>31</sup> The method established by Monden et al.<sup>23</sup> was used for developing retrotransposon-based DNA markers. First, genomic DNA of the  $F_1$  progeny lines and the parental cultivars was digested using gTUBE (Covaris Inc., MA, USA). Thereafter, adaptor ligation and PCR amplification of the insertion sites were performed using primers designed according to the retrotransposons and adaptors. PCR primers contained 5- or 7-bp barcode sequences for multiplexed sample analysis on the Illumina platform. The PCR library was gel-purified, and 400–600-bp fractions were selected using the MinElute Gel Extraction Kit (Qiagen). The purified product was qualified and quantified using the NanoDrop spectrophotometer and Qubit@2.0 fluorometer (Invitrogen). The MiSeq sequencing library was prepared by pooling equal amounts of purified barcoded products from each  $F_1$  line. The 150 bp of paired-end reads that were obtained were analysed using Maser, which is a pipeline execution system of the Cell Innovation Program at the National Institute of Genetics ([http://cell-innovation.nig.ac.jp/index\\_en.html](http://cell-innovation.nig.ac.jp/index_en.html) (date last accessed 17 July 2019)). Adaptor trimming using cutadapt<sup>32</sup> and quality filtering ( $QV \geq 20$ ) were performed. Filtered reads were trimmed to a specific length that covered most of the sequences. The reads with  $\geq 10$  identical sequences were collapsed into a single sequence in FASTA format and clustered using the BLAT alignment self-alignment program<sup>33</sup> with the following parameter settings:  $-\text{tileSize} = 8$ ,  $-\text{minMatch} = 1$ ,  $-\text{minScore} = 10$ ,  $-\text{repMatch} = -1$ , and  $-\text{oneOff} = 2$ . This clustering analysis produced several clusters

corresponding to a separate retrotransposon insertion site. An optimal threshold was set to evaluate the presence or absence of *Rtsp-1* and *Cl8* insertions: if the number reads on a given cluster or the total number of reads was  $<0.0001$ , then *Rtsp-1* or *Cl8* was considered not to have been inserted. Based on these criteria, we obtained *Rtsp-1* and *Cl8* insertion information (inserted, 1; not inserted, 0), which was used to provide molecular marker information for linkage analysis.

To develop expressed sequences tag (EST)-derived SSR markers, total RNA from the aerial parts (leaves and stems) and tubers of Fusanoaki was extracted using the Plant RNA Purification Reagent Kit (Invitrogen, CA, USA). Purification of polyadenylated RNA, conversion to cDNA, Sanger sequencing, and trimming were performed as previously described.<sup>34</sup> The fuzznuc program from EMBOSS<sup>35</sup> was used to identify SSRs longer than 14 bases that contained all possible combinations of di-, tri-, and tetranucleotide repeats and allowed a maximum of two mismatches in motifs. Primer pairs for the amplification of SSR-containing regions were designed based on the flanking sequences of each SSR, with the help of the Primer3 program,<sup>36</sup> in such a way that the amplified products ranged from 90 to 300 bp in length. PCR was performed with 0.6 ng of genomic DNA of the F<sub>1</sub> progeny lines and the parental cultivars in 1X PCR buffer (Bioline, UK), 3 mM of MgCl<sub>2</sub>, 0.08 units of BIOTAQ DNA polymerase (Bioline), 0.8 mM of dNTPs, and 0.4 μM of each primer. A modified touchdown PCR protocol was followed, as described by Sato et al.<sup>37</sup> Polymorphisms in PCR products were identified using 10% polyacrylamide gel electrophoresis in TAE buffer or with an ABI 3730xl fluorescent fragment analyzer (Applied Biosystems, USA), according to the polymorphic fragment sizes of the PCR amplicons. In the latter case, PCR amplicons were labelled with 10 μM of R6G-ddCTP or R110-ddUTP along with 0.16 units of Klenow fragments, 0.5 μl of 10X Klenow buffer (TAKARA BIO Inc., Shiga, Japan), and 0.16 units of Thermo Sequenase DNA polymerase (GE Healthcare UK Ltd, Buckinghamshire, England) before electrophoresis. The fragment sizes were investigated using Polyans software (<https://www.kazusa.or.jp/phenotyping/polyans/> (date last accessed 17 July 2019)) or GeneMarker software version 2.2.0 (SoftGenetics LLC, USA).

SNP markers were developed according to the methods of Shirasawa et al.<sup>27</sup> Briefly, the method for ddRAD-seq was as follows: Genomic DNA of the F<sub>1</sub> progeny lines and the parental cultivars was digested with two kinds of restriction enzymes (*Pst*I and *Msp*I). An adapter ligation reaction and gel purification were then performed, and a ddRAD-Seq library was developed. The HiSeq 2500 system (Illumina) was used for sequencing. Low-quality reads were removed, and adapter sequences were trimmed from the generated data using PRINSEQ (version 0.20.4)<sup>38</sup> and FASTX-Toolkit (version 0.013) ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/) (date last accessed 17 July 2019)). The reads were aligned with the genome sequence of *Ipomoea trifida* (version ITR\_r1.0),<sup>39</sup> a diploid wild relative of sweetpotato, using Bowtie 2.<sup>40</sup> SNP calling on the results of the alignment was performed using VarScan 2 (version 2.3)<sup>41</sup> and VCFtools.<sup>42</sup>

### 2.3. Linkage map construction

For linkage analysis in outcrossing species, such as sweetpotato, a two-way pseudo-testcross strategy developed by Grattapaglia and Sederoff (1994)<sup>43</sup> has been applied. In outcrossing species, dominant markers that are heterozygous in one parent and recessive homozygous in the other parent are segregated in the F<sub>1</sub> progeny; thus, analysing these markers develops two parental linkage maps. In addition, dominant markers that are heterozygous in both parents are segregated in the F<sub>1</sub> progeny, which can be used for linkage

analysis as well. In hexaploids, such as sweetpotato, several variations in segregation ratio can be detected based on the allele dosages (simplex, duplex, or triplex; Supplementary Table S1).<sup>44</sup> Alleles at higher dosages do not produce polymorphisms. These segregation ratios are affected by cytological characteristics (autohexaploid, tetradiploid, or allohexaploid; Supplementary Table S1). However, clear conclusions about the ploidy type of sweetpotato have not yet been reached.<sup>23</sup> Regardless of their cytological characteristics, only simplex markers that present as a single copy in one parent and are absent in the other parent segregate a 1:1 ratio (Supplementary Table S1). In addition, double simplex markers that present as a single copy in both parents segregate in a 3:1 ratio.<sup>19,21</sup> Thus, these simplex markers are highly useful for framework map construction.

The segregation ratio of the three types of molecular markers in the F<sub>1</sub> progeny was calculated, and a chi-square test ( $P \geq 0.01$ ) was performed with the calculated and expected segregation ratios. Markers with good fit were used to create a framework map. The linkage map was constructed using the OneMap software (version 2.0.4).<sup>45</sup> During linkage map creation, a logarithm of odds (LOD) score was used for the 'group' command to make the number of LGs equal to the number of sweetpotato chromosomes (90). For the 'order' command, parameters of `n.init = 5`, `subset.search = 'twopt'`, `twopt.alg = 'rcd'`, `THRES = 3`, `draw.try = TRUE`, `wait = 1`, and `touchdown = TRUE` were used. Imputation of missing values and erroneous genotyping data were performed using Maskov (version 1.0) with default parameters.<sup>46</sup> The imputed data were grouped and ordered again using OneMap software with the previously mentioned parameters. Graphical charts of the LGs were generated using MapChart software.<sup>47</sup>

### 2.4. Phenotyping of nematode resistance

Resistance tests were performed according to the method developed by Tabuchi et al.,<sup>18</sup> which is originally described by Sano and Iwahori.<sup>15</sup> We used three isolates of *M. incognita* [Nishigoshi (SP1), Tsukuba (SP4), and Okiishi-12 (SP6-1)], which were originally isolated by Sano et al.<sup>17</sup> and Sano and Iwahori.<sup>15</sup> The second-stage juveniles (J2) of *M. incognita* were freshly prepared for each resistance test as follows: a 19-day-old seedling of a susceptible tomato cultivar (Plitz) was inoculated in a 15-cm diameter pot containing ~600 g of a seeding culture soil (Kenbyo, Yaenogei, Japan) with ~6,000 J2, which remained from the former resistance test. Plitz was cultivated in a greenhouse at an average temperature of 25.5°C for 41–48 days. Egg masses, which nematodes formed on the tomato root systems during cultivation, were picked up and placed on a cotton filter partially submerged in water in a beaker at 24°C. In this system, J2 emerged from the egg masses, migrated through the filter, and accumulated at the bottom of the beaker. The cotton filter was transferred to a new beaker every 2–3 days, and the previous beaker was kept at 13°C, which is close to the developmental zero point of *M. incognita*.<sup>48</sup> J2 individuals were collected from 3–5 beakers and used for a new resistance test.

During resistance tests, sweetpotato individuals were cultivated at an average temperature of 25.5°C in a greenhouse. Each single-node cutting from F<sub>1</sub> progeny lines and parent cultivars produced roots in water in a beaker for 7–9 days and was then transferred into a 9-cm pot containing ~200 g of mixed soil (Kenbyo; steam-sterilized andosol = 1:1) and cultivated for 3 days. Each pot was inoculated with 500 J2 and cultivated for 3 days. The sweetpotato individuals were shaded with newspapers during these treatments because they did not have sufficient roots to be exposed to direct sunlight. Root systems were washed after 35 days of cultivation without shade, and egg masses were stained with 0.02% Erioglaurine (Sigma) and counted.

According to the previous reports,<sup>18,49</sup> we defined resistance as the presence of <10 egg masses per plant and susceptibility  $\geq 10$ . Resistance tests of F<sub>1</sub> progeny were performed on at least four plants. When the results of resistance or susceptibility were consistent among plants, the resistance test of the F<sub>1</sub> line was considered complete. When the results were different, resistance tests were continued. Finally, the resistance of F<sub>1</sub> progeny to each *M. incognita* race was tested on 4–29 plants, and the average number of egg masses was calculated. For QTL analysis, we used the phenotypic data obtained from 98, 103, and 98 F<sub>1</sub> lines for the SP1, SP4, and SP6-1 races, respectively, because the results of resistance tests from other lines were unstable.

## 2.5. QTL analysis and a GWAS

QTL analysis was performed using MapQTL 6.0.<sup>50</sup> Mixture Model was used as the analysis model, and interval mapping was performed using the remaining default settings. Furthermore, to calculate the LOD score as the threshold value for QTL detection, a permutation test (1,000 times) was performed with a significance level of 5%. In addition, to obtain results with higher reliability, GWAS was performed with the SNP marker genotype and nematode-resistance test results using TASSEL version 5.0.<sup>51</sup> To visualize the results of this study, a Manhattan plot was created using the qqman R package.<sup>52</sup> The Bonferroni correction for multiple testing was performed using an R script, and SNPs with a significantly high relationship to resistance were extracted.

## 2.6. Genotyping using SNP-based PCR markers

We attempted to develop a selection marker for nematode resistance found on the base sequences of the gene regions determined using QTL analysis and the GWAS. A primer with a J-Red SNP at the 3' end and an artificial mismatch at the third base were designed for PCR in accordance with the methods reported by Hayashi et al.<sup>53</sup> SNPs at and around the QTL peaks were used in the primer design. The genotyping primer sets were designed, and a preliminary genotype analysis was performed under the following conditions using only the parental and 14 F<sub>1</sub> progeny plants at first. The PCR reaction solution contained 10 ng of DNA, 1.0  $\mu$ l of 10X PCR Buffer, 0.8  $\mu$ l of dNTPs (2.5 mM each), 0.25 units of TAKARA *Taq* Hot Start Version, and 0.2  $\mu$ l each of the forward and reverse primers and was adjusted to a total volume of 10  $\mu$ l. The reaction was started at a temperature of 94°C for 2 min, and then cycled at 94°C for 10 s  $\rightarrow$  60°C for 10 s  $\rightarrow$  72°C for 30 s for 30 cycles and finished at 4°C. A 2% agarose gel was used for the detection of polymorphisms. A primer set that worked well for genotyping was selected, and genotype analysis was performed under the same conditions for the parental strains and the F<sub>1</sub> plants used for nematode resistance screening. The following primers derived from the Itr\_s002268\_68403 SNP were used for the genotyping of the parental plants and 104 F<sub>1</sub> progeny plants: forward: 5'-GCTTCGGCTCCACGTC AAT and reverse: 5'-CCAACAACACTCCAAAGCAAG.

## 3. Results

### 3.1. High-density linkage map construction using SNP, SSR, and retrotransposon-based markers

According to the sequencing procedure that develops retrotransposon-based markers, 30,372,493 150-bp paired-end reads, including exact barcode sequences, were obtained using MiSeq, with 17,649,817 remaining reads, representing 42,059 individual sequences (around

420-fold sequence redundancy), after pre-processing events, such as trimming and filtering. Results of the clustering analysis using BLAT identified *Rtsp-1* insertions in 222 sites in J-Red, 207 sites in Choshu, and an average of 202.5 sites in the F<sub>1</sub> mapping populations. *Cl8* family insertions in 610 sites in J-Red, 613 sites in Choshu, and an average of 564.6 sites in the F<sub>1</sub> mapping populations were identified. For the identification of insertion sites, the allele dosage of an insertion was considered from a chi-square goodness-of-fit test ( $P \geq 0.01$ ) with the expected segregation ratio. For *Rtsp-1*, of the 105 sites that existed in both parents, 41 (39.0%) were believed to be double simplex. The insertion sites that existed only in either J-Red or Choshu occurred at 117 and 102 sites, respectively, and 99 (84.6%) and 79 (77.5%) sites were believed to be simplex insertions, respectively. For *Cl8*, of the 318 sites that existed in both parents, 118 (37.1%) were believed to be double simplex. The insertion sites that existed only in either J-Red or Choshu occurred at 292 and 295 sites, respectively, and 182 (62.3%) and 173 (58.6%) sites were believed to be simplex insertions, respectively. In total, 281 and 252 insertion sites were considered simplex markers in J-Red and Choshu, respectively, and 159 insertion sites were considered double simplex markers (Table 1).

For the development of EST-derived SSR markers, a total of 7,680 cDNA clones were sequenced, which include 3,840 clones each from the aerial and tubers of the sweetpotato cultivar Fusanoaki. A sum of 7,669 ESTs was obtained after trimming. Of the 7,669 ESTs, 564 SSRs were identified, and the primers were successfully designed on the flanking regions of the 121 SSRs (Supplementary Table S2). To increase the number of EST-SSR markers, additional primer pairs were designed that allowed either single- or two-base mismatches. The newly developed markers were designated as *Ipomoea* EST-derived SSR (IES) markers. As a result, a total of 1,215 IES markers were designed (Supplementary Table S2). Among the 1,215 IES markers generated, 978 (80.5%) were trinucleotide repeats, whereas 133 (10.9%) and 104 (8.6%) were di- and tetranucleotide repeats, respectively. The poly-(AAG)<sub>n</sub> motif was most abundant in the trinucleotide repeats, followed by poly-(GGC)<sub>n</sub> and poly-(GGA)<sub>n</sub>. Among the di-nucleotide motifs, poly-(AG)<sub>n</sub> was the most frequently observed. The primer sequences and details of the IES markers are described in Supplementary Table S3. The 1,215 IES markers were subjected to polymorphic marker screening by electrophoresis on 10% acrylamide gels using the DNAs of both parents and the F<sub>1</sub> mapping populations. The results for SSR marker genotype information showed that 632 markers existed in both parents, 335 in J-Red, and 418 only in Choshu. The results of the goodness-of-fit test with the expected segregation ratio showed that 143 (22.6%) of all the markers that existed in both parents were believed to be double simplex and 181 (54.0%) and 219 (52.4%) of the markers that existed only in J-Red and Choshu were believed to be simplex, respectively (Table 1).

After sequencing the ddRAD-seq library with the HiSeq 2500 platform, a total of 263,837,348 reads were obtained, with an average of 2.4 million reads per plant. We identified 56,513 candidate SNPs by comparing the reads obtained from a ddRAD-Seq library sequence analysis with the *I. trifida* genome sequence.<sup>39</sup> We can assume seven different genotypes representing genetic loci in a hexaploid as follows: AAAAAA, AAAAAa, AAAAaa, AAAaaa, AAaaaa, Aaaaaa, and aaaaaa. 'A' is identical to the *I. trifida* reference allele, and 'a' is different from the *I. trifida* reference allele. Among them, only simplex or double simplex alleles were extracted using a chi-square goodness-of-fit test ( $P \geq 0.01$ ). A test for goodness-of-fit with the expected segregation ratio was conducted, and the results indicated that there were 2,418 (AAAAAa  $\times$  AAAAAa) and 485 (Aaaaaa  $\times$  Aaaaaa) SNPs that there were believed to be double simplex alleles in both parents. In total, 2,903 SNPs were extracted as

**Table 1.** Summary of molecular markers developed for linkage map construction

Marker types	Simplex type	Expected segregation ratios	No. of selected markers ( $P \geq 0.01$ )	Total
SNP	J-Red	1:1	5,563	13,575
	Choshu		5,109	
	Both parents	3:1	2,903	
SSR	J-Red	1:1	181	543
	Choshu		219	
	Both parents	3:1	143	
Retrotransposon-based	J-Red	1:1	281	692
	Choshu		252	
	Both parents	3:1	159	

double simplex markers for further analysis. In SNPs that existed only in J-Red, there were 4,885 (AAAAAA × AAAAAa) and 678 (aaaaaa × Aaaaaa) SNPs that were believed to be simplex alleles; meanwhile, in SNPs that only existed in Choshu, there were 4,502 (AAAAAA × AAAAAA) and 607 (Aaaaaa × aaaaaa) SNPs that were considered to be simplex alleles. In total, there were 5,563 (4,885 + 678) and 5,109 (4,502 + 607) simplex SNP markers in J-Red and Choshu cultivars, respectively (Table 1). These three types of marker that had a good fit with the expected segregation ratio (retrotransposon-based, SSR, and SNP) were used in the linkage analysis (Table 1).

A linkage map was constructed using the OneMap program<sup>45</sup>; the linkage maps for J-Red and Choshu cultivars were both created with 90 LGs. There were 6,341 markers in J-Red (SNP: 5,952, retrotransposon-based: 228, SSR: 161), yielding a linkage map with a total length of 13,247.0 cM (Table 2 and Supplementary Table S4). The average length of each linkage group was 147.2 cM, the average distance between markers was 2.09 cM, and the average number of markers was 70.5 (Table 2). There were 6,008 markers in Choshu (SNP: 5,640, retrotransposon-based: 192, SSR: 176), yielding a linkage map with a total length of 12,241.8 cM (Table 2 and Supplementary Table S5). The average length of each linkage group was 136.0 cM, the average distance between markers was 2.04 cM, and the average number of markers was 66.8 (Table 2). The LGs were named in accordance with the map reported by Shirasawa et al.<sup>27</sup> by anchoring common SNP positions on the ITR\_r1.0.

### 3.2. Phenotyping of nematode resistance

The results of the evaluation of *M. incognita* resistance are shown in Fig. 1. For the parental cultivars, the average number of egg masses on J-Red (0.4, 1.9, and 1.3 for the SP1, SP4, and SP6-1 races, respectively) was significantly smaller than on Choshu (178.1, 233.0, and 209.6 for the SP1, SP4, and SP6-1 races, respectively); there was a large difference in the resistance of the parental cultivars. When defining resistance as <10 egg masses and susceptibility as ≥10 egg masses,<sup>18,49</sup> the F<sub>1</sub> progeny segregated a 1:1 ratio according to resistance or susceptibility to any of the races (Supplementary Table S6). Segregation of egg mass numbers for the F<sub>1</sub> progeny was extremely biased and did not follow a normal distribution; as a result, they were not suitable for QTL analysis. Thus, a value of 1 was added to the number of egg masses, and the common logarithm was taken to yield the input value used for QTL analysis. The reason why a value of 1 was added was that the average number of egg mass was zero in some F<sub>1</sub> plants and the common logarithm of zero is not defined. In addition, the correlation between each race was investigated using these values after logarithmic conversion. The correlations between

**Table 2.** Summary of linkage groups constructed in this study

Parent	J-Red	Choshu
No. of linkage maps	90	90
No. of mapped markers	6,341	6,008
Total length (cM)	13,247	12,242
Average length of linkage groups (cM)	147.2	136.0
Average number of markers among the linkage groups	70.5	66.8
Marker density (cM)	2.09	2.04

SP1 and either SP4 or SP6-1 were 0.77 and 0.86, respectively, and the correlation between SP4 and SP6-1 was 0.75; all these are high values (Supplementary Fig. S1). Consequently, resistance to these three races was found to be highly correlated.

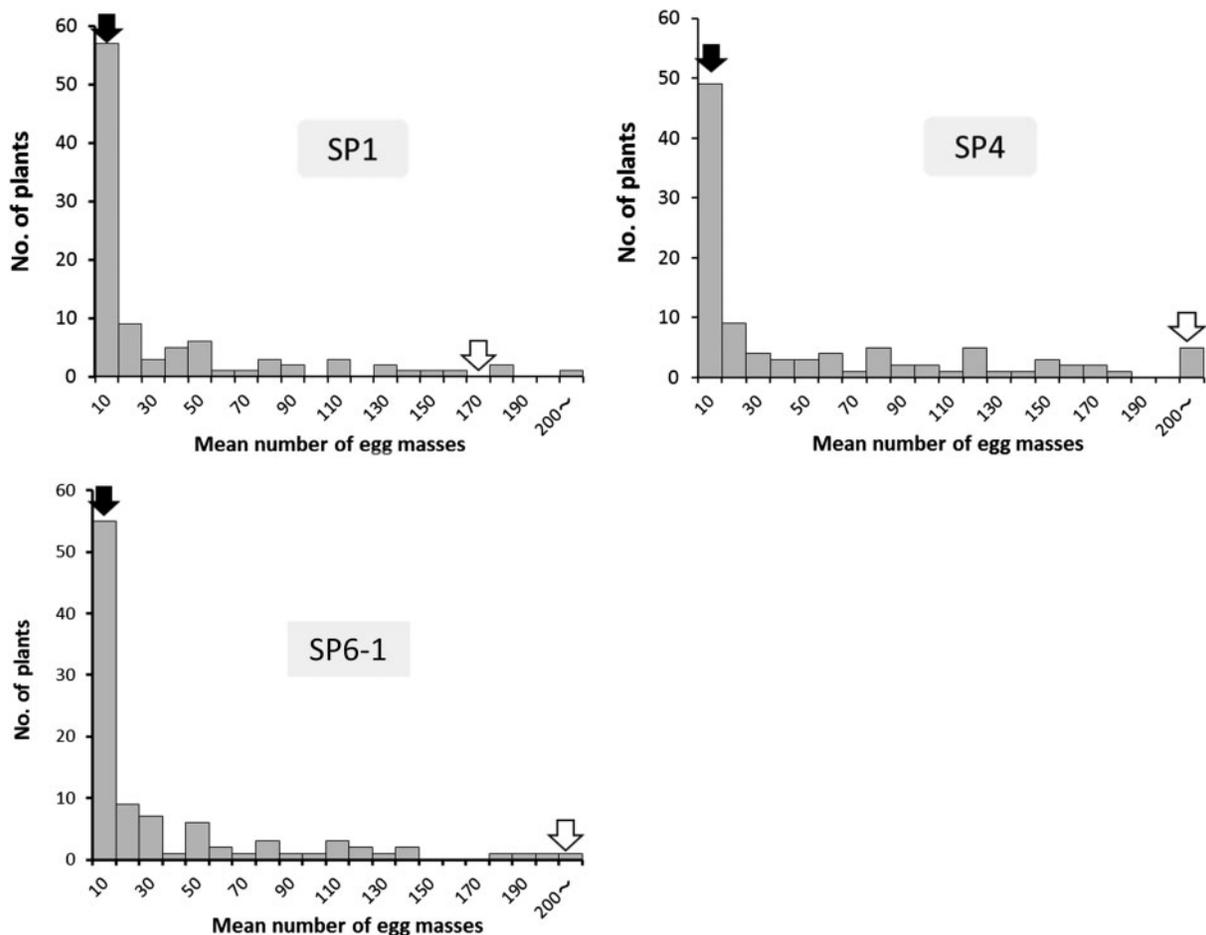
### 3.3. QTL analysis and GWAS

QTL analysis of resistance to SP1, SP4, and SP6-1 was conducted using the high-density linkage map. Regions with an LOD score of ≥2.5 based on the analysis of MapQTL 6 were considered as candidate QTL peaks. The LOD threshold was determined with 1,000 permutations. The results indicated that the candidate QTL peaks (explaining 29.6–36.7% of the variance) for the highly effective resistance of J-Red to all the races were found in the Ib01\_6 linkage group (Table 3). All these are located extremely close together in a region on the linkage group, and based on the high correlation between the three races, this region is thought to be a common QTL. Other small QTL peaks included an SP4-susceptible candidate QTL (explaining 30.0% of the variance) detected in Ib01\_4 in J-Red, an SP4-resistant candidate QTL (explaining 21.7% of the variance) in Ib07\_4 in J-Red, and an SP6-1-susceptible candidate QTL (explaining 26.6% of the variance) on Ib01\_1 in Choshu (Table 3).

In addition, GWAS was conducted using the resistance test results and SNP marker genotype data. One peak that was highly correlated to resistance to all races was found on Ib01\_6 (Fig. 2). This peak is consistent with the major QTL peak on Ib01\_6 that was detected via QTL analysis (Supplementary Table S7); thus, it is believed to be effective. Accordingly, we developed a PCR-based DNA marker for *Meloidogyne* resistance selection with a focus on this QTL.

### 3.4. Development of PCR-based DNA markers for selecting resistant plants

We focussed on three SNP markers that exist near the QTL peak on Ib01\_6 (Itr\_sc002268\_68403, Itr\_sc003013\_33365, and Itr\_sc000186\_110963) to develop a marker for the selection of resistance to SP1, SP4,



**Figure 1.** Frequency distribution of the mean number of egg masses from races SP1, SP4, and SP6-1 of the nematode *Meloidogyne incognita* on  $F_1$  plants from a cross between the resistant cultivar J-Red and the susceptible cultivar Choshu in sweetpotato. Black and white arrows indicate the values of J-Red and Choshu, respectively.

**Table 3.** Summary of QTLs identified in this study

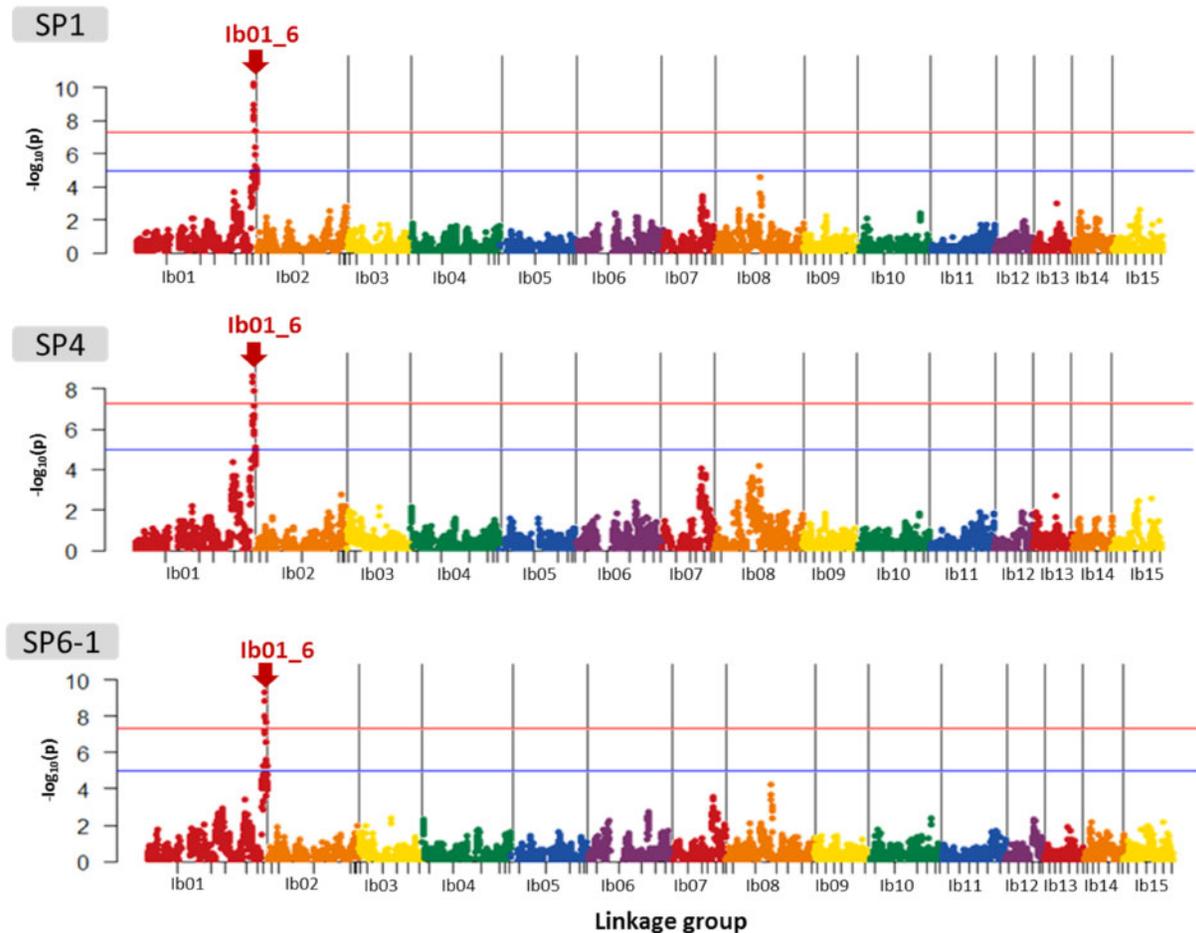
Race	Parent	Linkage group	QTL peak position (cM)	LOD score	Nearest marker	$R^2$ (%)	Effect
SP1	J-Red	Ib01_6	74.39	9.37	Itr_sc002268_68403	36.7	R
SP4	J-Red	Ib01_4	120.23	3.47	Itr_sc000224_134385	30.0	S
		Ib01_6	76.25	7.71	Itr_sc001883_69062	29.6	R
SP6-1	J-Red	Ib07_4	67.75	4.00	Itr_sc001596_10966	21.7	R
	Choshu	Ib01_6	81.02	8.26	Itr_sc003013_33365	32.7	R
		Ib01_1	34.41	3.59	Itr_sc000336_34336	26.6	S

and SP6-1. First, we designed PCR primers for genotyping according to the methods developed by Hayashi et al.<sup>53</sup> These were designed based on J-Red-derived SNPs. Using these primers, we conducted a pilot study on the parental cultivars and 14  $F_1$  progeny. The clearest bands were detected when using primers based on Itr\_sc002268\_68403 (Supplementary Fig. S2). In addition, as expected, a clear single band was observed in J-Red, but not in Choshu. Thus, we used this primer to conduct PCR genotyping of the parental cultivars and 104  $F_1$  progeny (Fig. 3 and Supplementary Fig. S3). The mean number of egg masses on the  $F_1$  plants was significantly different based on the genotypes of Itr\_sc002268\_68403 SNP, which indicates that this marker had the greatest effect on resistance (Fig. 4). Genotyping results using this marker showed that of the 104 progeny, resistance or susceptibility was

discernible in 74 (71.2%) for SP1, 72 (69.2%) for SP4, and 76 (73.0%) for SP6-1 (Supplementary Fig. S3). Using the marker we developed, whether a sweetpotato plant is resistant or susceptible to the three races of nematodes can be identified with a high accuracy rate at 70%. Therefore, this marker can be used for discerning resistance to multiple races of *M. incognita* during marker-assisted selection of sweetpotato.

#### 4. Discussion

In this study, a high-density linkage map of the sweetpotato genome was successfully established using SNP, retrotransposon, and SSR markers. In addition, QTLs related to *M. incognita* resistance were



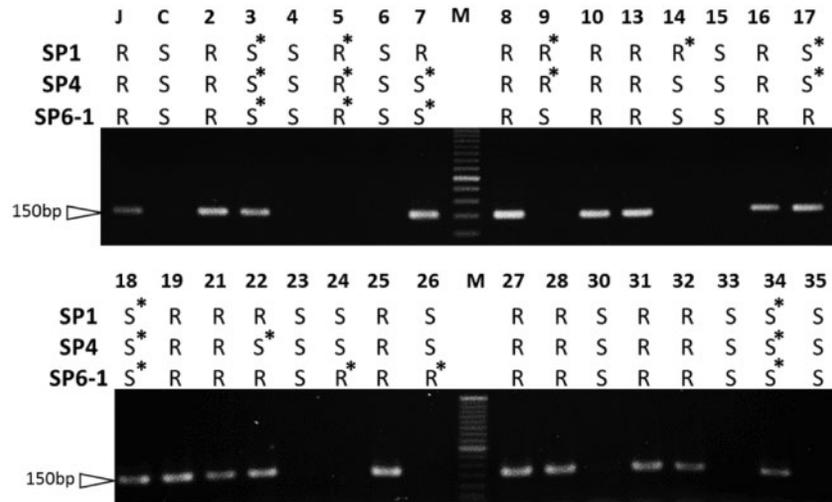
**Figure 2.** Manhattan plots for sweetpotato resistance to the three races of the nematode *Meloidogyne incognita* (SP1, SP4, and SP6-1). X-axis: the linkage groups from lb01-1 to lb15-6 (each linkage group is separated by a line, and detailed information about the J-Red linkage groups are shown in [Supplementary Table S4](#)); Y-axis:  $-\log_{10}(P)$ . The lower horizontal line represents the Benjamini-Hochberg corrected significance threshold. The upper horizontal line represents the Bonferroni corrected significance threshold. AQ15

detected using this linkage map. Therefore, we were able to discern resistance from susceptibility, with a high level of probability, by genotyping  $F_1$  plants using PCR primers based on the identified SNPs. Thus, the marker we developed in this study is likely to be useful in selective breeding for resistance to multiple races of *M. incognita*. This study provides a breakthrough in the development of selective markers found on NGS-based comprehensive genetic analyses in sweetpotato, a highly polyploid and non-model crop species lacking reference genome sequences.

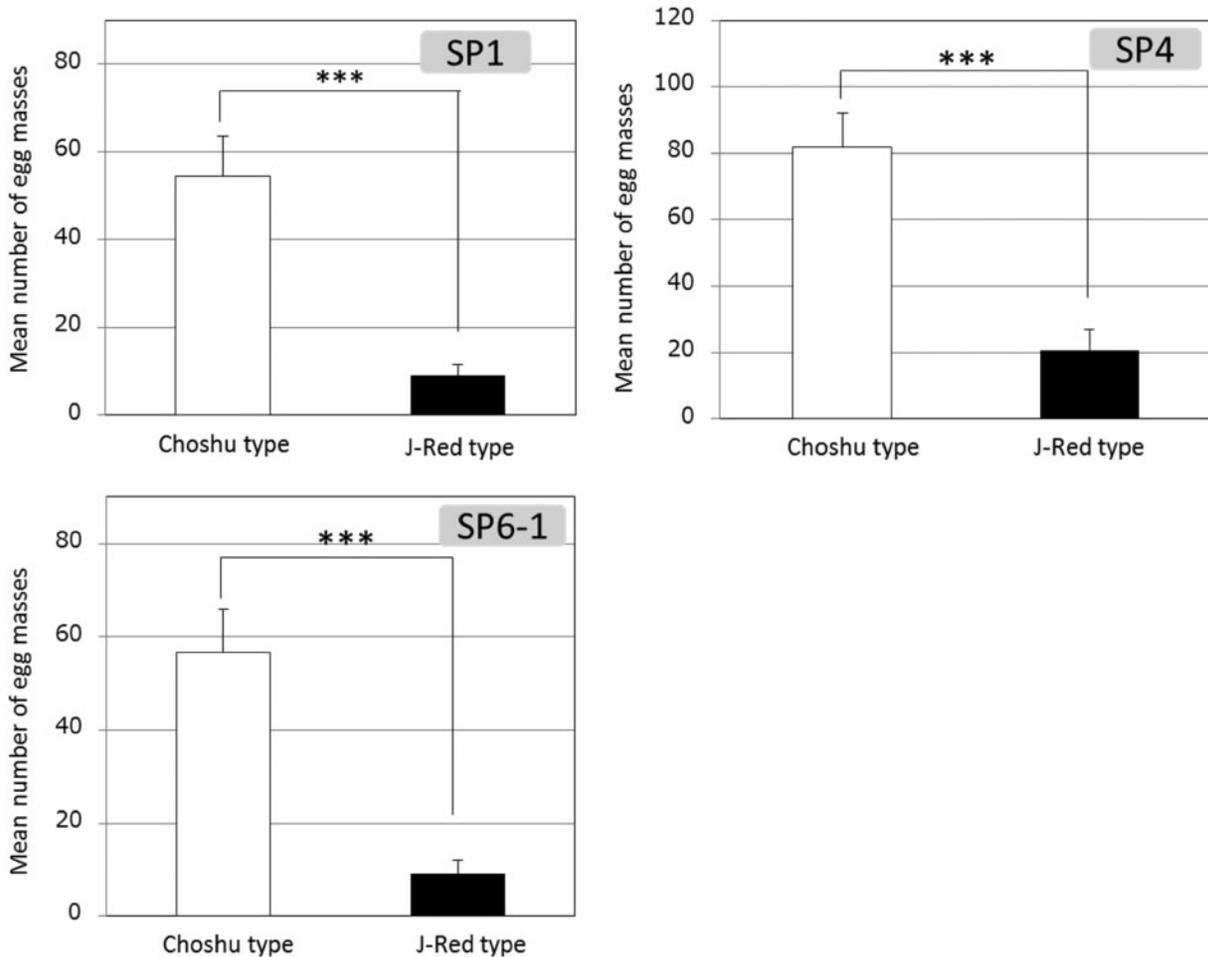
There are other reports of QTL analysis in *M. incognita* and the development of resistance markers. Whether resistance to *Meloidogyne* is a qualitative or quantitative trait is still open for debate, and to date, a definitive answer has not been reached.<sup>54–57</sup> Cervantes-Flores et al.<sup>58</sup> have created a linkage map and identified nine QTLs related to *M. incognita* in sweetpotato, but these showed only small effects. On the contrary, two independent dominant genes (*Rmi1* and *Rmi2*) have been reported to control *Meloidogyne* resistance in *I. trifida*, the ancestral species of sweetpotato.<sup>59,60</sup> In addition, a recent report by Nakayama et al.,<sup>49</sup> which conducted a QTL analysis of the  $F_1$  population of a hybrid of the SP1/SP2-resistant cultivar Hi-starch and the nematode-susceptible cultivar Koganesengan, indicated that SP1/SP2 resistance in Hi-starch was controlled by a single major dominant gene that showed simplex inheritance. Their

data have indicated that the positions of the genes controlling SP1/SP2 resistance are either the same or extremely similar. In addition, the AFLP markers E33M53\_090 and E41M32\_206 around this resistance gene location (*qRmi(t)*) were converted to sequence-characterized amplified region (SCAR) markers. In the present study, J-Red-derived SP1-, SP4-, and SP6-1-resistant QTLs were identified using the  $F_1$  progeny of the resistant cultivar J-Red and the susceptible cultivar Choshu, and we developed a marker that facilitates the use of SNPs in these regions for simple genotyping. To investigate the consistency between the Hi-starch-derived QTL and the J-Red-derived QTL, primer sequences SCAR markers developed by Nakayama et al.<sup>49</sup> were blasted against the genome sequences of *I. trifida* (version ITR\_r1.0). Results showed that the primer sequences had no hits with any scaffolds located in the J-Red-derived QTL region. Thus, the J-Red-derived QTL identified in our study should be novel. The dominant race of nematode in sweetpotato fields varies according to the cultivation area; therefore, ideal markers that can be used to identify resistance to multiple races will be established. In this study, we developed a marker that can be used for the selection of sweetpotato cultivars that are resistant to three races of nematode.

*Mi-1* gene was reported to belong to the class of nucleotide binding site-leucine rich repeat (NBS-LRR) resistance (R) genes confers resistance to *M. incognita* in tomato.<sup>61</sup> We attempted to identify the



**Figure 3.** PCR genotyping using the developed DNA marker derived from the *Itr\_sc002268\_68403* SNP. J: J-Red; C: Choshu; 2-35: F<sub>1</sub> progeny; and M: 100-bp DNA ladder. The evaluation of the resistance to SP1, SP4, and SP6-1 races of the nematode *Meloidogyne incognita* is shown on top of each lane. R and S indicate resistance and susceptibility, respectively. \*The reaction (resistance/susceptibility) is not consistent with the presence or absence of bands.



**Figure 4.** The mean number of egg masses for the three races of the nematode *Meloidogyne incognita* on F<sub>1</sub> plants from a cross between the resistant cultivar J-Red and the susceptible cultivar Choshu in sweetpotato grouped by the *Itr\_sc002268\_68403* marker genotype. \*\*\*Significant differences at  $P < 0.001$  based on a two-tailed *t*-test.

target gene(s) controlling *M. incognita* resistance in the QTL region on the Ib01-6 linkage group. A total of 254 genes in the *I. trifida* scaffolds corresponded to this QTL region. We found that one gene (Itr\_sc000186.1\_g00004.1) belongs to the NBS-LRR family based on the gene annotation information. However, the Itr\_sc000186.1 scaffold is positioned slightly apart from the region with the highest QTL peak, and this scaffold exists in three LGs (Ib01-1, Ib01-4, and Ib01-6). One of the possibilities is that this gene might exist on the three homologous genomes (Ib01-1, Ib01-4, and Ib01-6), and only the allele on the Ib01-6 linkage group may affect resistance. To validate the target gene(s) for resistance, information from ongoing projects on deciphering the whole genome sequences and the haplotype phasing of the hexaploidy species *I. batatas* is awaited.<sup>62</sup> In addition, we are currently conducting an RNA-seq analysis using J-Red and Choshu cultivars with or without nematode inoculation. Considering the data from the differentially expressed genes (DEGs), this QTL region may facilitate cloning of the target gene(s).

Evaluation of resistances to SP1, SP4, and SP6-1 showed a high correlation in the F<sub>1</sub> mapping populations of J-Red and Choshu (Supplementary Fig. S1). In fact, we detected a highly effective QTL that was common for resistance in all three races in the Ib01-6 linkage group (Fig. 2 and Table 3). However, the reaction (resistant/susceptible) of each F<sub>1</sub> line to the three races was not completely consistent (Supplementary Fig. S3). Moreover, several other minor QTLs were detected for races SP4 and SP6-1, which should affect resistance (Table 3). Thus, we evaluated the effectiveness of these minor QTLs and the combinations of multiple QTLs. SP4-susceptible and -resistant QTLs were also detected on Ib01-4 and Ib07-4 in J-Red, which is the resistant cultivar, respectively (Supplementary Figs S4 and S5). We designed these QTLs on Ib01-6, Ib07-4, and Ib01-4 as R1, R2, and S, respectively. We divided the plants into eight groups (=2<sup>3</sup>) based on the presence or absence of the combinations of these detected QTLs. We compared the average number of egg masses found on these eight groups in the F<sub>1</sub> plants (Supplementary Fig. S6). The average number of egg masses on plants with J-Red genotypes for the two resistant QTLs and the Choshu genotype for the susceptible QTL (R1 × R2 × s) was 7.7 (Supplementary Fig. S6). Moreover, this group had the highest proportion of resistant plants (13/16 = 81.3%). By contrast, the group of plants that had Choshu genotypes for the two resistant QTLs and the J-Red genotype for the susceptible QTL (r1 × r2 × S) had the greatest proportion of susceptible plants (resistance in 2/20 plants = 90.0% susceptibility). Interestingly, the average number of egg masses on the plants with the R1 × r2 × S genotype was 60.1, whereas that on the plants with the R1 × R2 × S genotype was 8.6, which indicated that R2 should suppress the effect of S only when R2 coexists with R1. Although the target genes of those QTLs have not been resolved and the details are unknown, a genetic interaction between R1 and R2 might exist. Based on the abovementioned information, the genotypes at these three QTLs could be used to obtain more detailed information for the selection of resistance or susceptibility. However, as the number of lines decreases when classifying them according to the presence of three specific genotypes, it may be most effective to use the major QTL on Ib01-6 as a marker for selection. In addition, the number of egg masses on the plants with the R1 × R2 × S genotype (8.6) was slightly higher than on J-Red (1.9), which suggested that other undetected minor QTLs may affect the resistance. While a QTL believed to be involved in susceptibility to SP6-1 was detected in Choshu, there was no significant difference in the number of egg masses between strains with or without the Choshu genotype for this QTL (Supplementary Fig. S7).

J-Red has a rare characteristic of resistance to almost all races of *M. incognita*, except for SP8. Therefore, this cultivar may be used for QTL analysis and for the development of other markers for the selection of resistant cultivars against other races of nematodes that were not used in this study. We are currently working on QTL analysis and the development of markers for the selection of resistance to other races of nematodes. Our research has shown that it is possible to use NGS to develop high-density linkage maps and for genome-wide analysis of genetically complex crop species, such as sweetpotato; these can be used for the identification of more effective QTLs and the development of markers for selection. As genomic information continues to improve, genes related to resistance may be detected in the near future.

## Availability

The ESTs obtained to develop SSR markers were submitted to the DDBJ/EMBL/GenBank databases under the accession numbers HX972900 to HX980564. The MiSeq reads for analysing retrotransposon insertion sites were under the accession number DDBJ: DRA005090. The HiSeq reads of ddRAD-seq were under the accession number DDBJ: DRA006994.

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## Accession numbers

HX972900, HX980564, DRA005090, DRA006994

## Conflict of interest

None declared.

## Supplementary data

Supplementary data are available at DNARES online.

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