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Research Paper

Fine mapping of the gene for susceptibility to black spot disease in Japanese pear (*Pyrus pyrifolia* Nakai)

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Black spot disease, which is caused by the Japanese pear pathotype of the filamentous fungus *Alternaria alternata* (Fries) Keissler, is one of the most harmful diseases in Japanese pear cultivation. We mapped a gene for susceptibility to black spot disease in the Japanese pear (*Pyrus pyrifolia* Nakai) cultivar 'Kinchaku' (*Aki* gene) at the top of linkage group 11, similar to the positions of the susceptibility genes *Ani* in 'Osa Nijisseiki' and *Ana* in 'Nansui'. Using synteny-based marker enrichment, we developed novel apple SSR markers in the target region. We constructed a fine map of linkage group 11 of 'Kinchaku' and localized the *Aki* locus within a 1.5-cM genome region between SSR markers Mdo.chr11.28 and Mdo.chr11.34. Marker Mdo.chr11.30 co-segregated with *Aki* in all 621 F_1 plantlets of a 'Housui' × 'Kinchaku' cross. The physical size of the *Aki* region, which includes three markers (Mdo.chr11.28, Mdo.chr11.30, and Mdo.chr11.34), was estimated to be 250 Kb in the 'Golden Delicious' apple genome and 107 Kb in the 'Dangshansuli' Chinese pear genome. Our results will help to identify the candidate gene for susceptibility to black spot disease in Japanese pear.

Key Words: *Alternaria alternata*, black spot disease, *Pyrus pyrifolia*, fine mapping, comparative genomics.

Introduction

Pears (*Pyrus* spp.) have been cultivated in East Asia, Europe, and North America for more than 3000 years and are among the most important fruits in over 50 countries in temperate zones (Bell 1990, Bell *et al.* 1996). The Japanese pear (*Pyrus pyrifolia* Nakai), European pear (*P. communis* L.), and Chinese pears (*P. bretschneideri* Rehd. and *P. ussuriensis* Maxim.) are the major edible species commercially grown for fruit production (Bell *et al.* 1996). The Japanese and Chinese pears are cultivated in East Asia, and the European pear is grown in Europe, North America, and temperate regions of the southern hemisphere. All *Pyrus* species are intercrossable and there are no major incompatibility barriers to interspecific hybridization in this genus (Westwood and Bjornsta 1971).

Black spot disease, caused by the Japanese pear pathotype of the filamentous fungus *Alternaria alternata* (Fries) Keissler (previously *A. kikuchiana* Tanaka), is one of the most serious diseases of Japanese pear cultivated in Asia. Previously, black spot disease was not observed in North America or Europe (Sanada *et al.* 1988). However, this disease had already occurred in 'Nijisseiki' pear orchard in Europe (Baudry *et al.* 1993, Kohmoto *et al.* 1992). Bagging and fungicide spraying to prevent infection by *A. alternata* are costly and labor-intensive (Kozaki 1973). *Alternaria* fungi produce host-selective toxins (HSTs), the structures of which have been determined in Japanese pear, strawberry, tangerine, apple, tomato, and rough lemon pathotype (Tsuge *et al.* 2013, Walton 1996). The AK-toxin, which is specifically produced by the Japanese pear pathotype, causes necrosis, early leaf fall, and low yield in Japanese pears, especially in commercial cultivars 'Nijisseiki', 'Shinsui', and 'Nansui' (Nakashima *et al.* 1985). The range of pear cultivars sensitive to AK-toxin is the same as the host range of the pathogen (Otani *et al.* 1985). Susceptibility to black spot disease is controlled by the single dominant gene *A* (Kozaki 1973). Susceptible cultivars are heterozygous (*A*/*a*), and homozygous (*A*/*A*) cultivars have not been identified. The inactivation of this gene has been attempted to induce disease-resistant mutant. A resistant mutant, γ-1-1 (current cultivar name 'Gold Nijisseiki'), was selected from 'Nijisseiki' plants after chronic irradiation of gamma-rays (Sanada *et al.* 1988).

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Banno *et al.* (1999) identified random amplified polymorphic DNA (RAPD) markers associated with susceptibility to black spot in the Japanese pear 'Osa Nijisseiki'; one of these markers, CMNB41, was linked to the susceptibility gene at a genetic distance of 3.1 cM. 'Kinchaku', an old Japanese pear cultivar of unknown parentage, is also susceptible to black spot. A genetic linkage map of 'Kinchaku' was constructed with RAPD markers (120 loci in 18 linkage groups), and the susceptibility gene was mapped (Iketani *et al.* 2001). However, these studies did not anchor these linkage groups to the reference linkage maps, and little information was available on molecular markers linked to the gene. In a previous study, we obtained simple sequence repeat (SSR) markers linked to the susceptibility genes in the Japanese pear cultivars 'Osa Nijisseiki' (gene *Ani*) and 'Nansui' (gene *Ana*) (Terakami *et al.* 2007). We used two mapping populations derived from crosses between 'Osa Nijisseiki' and 'Okusankichi' (110 F_1 plantlets) and between 'Oushuu' and 'Nansui' (40 F_1 plantlets) for genetic analysis of susceptibility to black spot. The SSR markers CH04h02 and CH03d02 were tightly linked to both *Ani* and *Ana*. Although *Ani* and *Ana* are derived from different cultivars, both genes are located at the top of linkage group 11 (Terakami *et al.* 2007).

Pears and apples (*Malus* × *domestica* Borkh.) belong to the family Rosaceae, subfamily Amygdaloideae, tribe Pyreae, and the two species have the same basic chromosome number ($x = 17$). The family Rosaceae includes many well-known and beloved species of economic importance, in particular fruits (Janick 2005) and ornamentals, but also some timber crops and many medicinal and nutraceutical plants (Hummer and Janick 2009). It includes from 95 to >100 genera and 2830–3100 species (Judd *et al.* 1999, Mabberley 1987). Velasco *et al.* (2010) reported a high-quality draft genome sequence of the domesticated apple. A hybrid assembly of Sanger and 454 reads produced 122,146 contigs, 103,076 of which were assembled into 1,629 metacontigs. The total length of available contigs (603.9 Mb) covers about 81.3% of the estimated apple genome size (742.3 Mb). Whole genome sequences have also been reported for Chinese pear (Wu *et al.* 2013) and European pear (Chagné *et al.* 2014). The draft genome sequence of the Chinese pear was determined using a combination of BAC-by-BAC and next-generation sequencing, and the assembled genome consists of 2103 scaffolds with an N50 of 540.8 Kb, totaling 512.0 Mb with 194× coverage, close to the estimated size of 527.0 Mb (Wu *et al.* 2013). Transferability of SSR information has been reported within the Pyreae (previously Maloideae *sensu lato*) (Liebhard *et al.* 2002, Silfverberg-Dilworth *et al.* 2006, Yamamoto *et al.* 2001), indicating the collinear synteny between pear and apple in all 17 linkage groups. These published genome sequences of the Pyreae could support and accelerate the genome analysis, including marker development and gene prediction, in Japanese pear.

Alternaria blotch, caused by the apple pathotype of *A. alternata* (previously *A. mali* Roberts), is an economically important disease in apple production in Japan and other Asian countries. Saito and Takeda (1984) suggested that susceptibility to Alternaria blotch in apple might be controlled by a single dominant gene, *Alt*, and that the most susceptible cultivars are heterozygous. SSR markers linked to the susceptibility genes of 'Starking Delicious' and 'Golden Delicious' have been reported (Li *et al.* 2011, Moriya *et al.* 2013).

In this study, we constructed a genetic map of linkage group 11 of 'Kinchaku' and identified the exact position of the gene for susceptibility to black spot disease using a large mapping population derived from a 'Housui' (syn. 'Hosui') × 'Kinchaku' cross. 'Housui', a leading Japanese pear cultivar, is resistant to black spot disease and has a homozygous recessive genotype (*a*/*a*), whereas 'Kinchaku' is susceptible and has a heterozygous genotype (*A*/*a*). We refer to the susceptibility gene of 'Kinchaku' as *Aki*; therefore, the genotype of 'Kinchaku' was considered as *Aki*/*a*. Using a comparative genomic approach, we developed a number of apple SSR markers in the region containing the susceptibility gene *Aki* and used them to fine-map the gene. Novel markers tightly linked to *Aki* were obtained, and one of them co-segregated with *Aki* in 621 F_1 plantlets.

Materials and Methods

Plant materials and DNA extraction

A mapping population was derived from a cross between Japanese pear cultivars 'Housui' and 'Kinchaku' and included 621 F_1 plantlets. Ungrafted progeny was grown in plastic pot (18 cm diameter × 16 cm height), and maintained in the greenhouse at the NARO Institute of Fruit Tree Science (Tsukuba).

Frozen young leaves (50 mg) were homogenized for 20 s in a Shake Master Auto (Bio Medical Science). Genomic DNA was extracted with a NucleoMag 96 Plant kit (Macherey-Nagel) according to the manufacturer's instructions, except that lysis buffer MC1 contained 2% 2-mercaptoethanol.

Evaluation of black spot susceptibility or resistance

Responses to black spot disease were evaluated by using the spore inoculation test (Hayashi *et al.* 1990). Both parental cultivars and all F_1 progeny were inoculated with spores of the virulent isolate No. 15A of *A. alternata*, which was kindly provided by Dr. T. Tsuge (Nagoya University). The isolate was cultured in potato dextrose broth without shaking for 10 days at 25°C. Mycelial mats were washed with tap water to remove culture medium and maintained at 25°C in the dark. The spores formed were collected, suspended in distilled water, and diluted to a concentration of approximately 5×10^5 spores/mL. The spore suspension was sprayed with a glass atomizer onto two young leaves detached from each plant. The inoculated leaves were incubated in a moist chamber at 25°C for 48 h. Leaves were classified into two groups: resistant (no disease symptoms) and

susceptible (necrotic symptoms). All inoculation tests were duplicated for all plantlets.

Positional identification of the susceptibility gene Aki

Because the black spot susceptibility genes *Ani* and *Ana* were both located in the same region of linkage groups 11, we suspected that *Aki* might also be in that region. To confirm this, all F_1 plantlets of the 'Housui' × 'Kinchaku' cross were analyzed using SSR markers CH04h02 and CH03d02 (mapped on linkage group 11), which show significant linkage to both *Ani* and *Ana* (Terakami *et al.* 2007).

A total of 18 SSR markers previously mapped on linkage group 11 of pear or apple (Liebhard *et al.* 2002, Terakami *et al.* 2014) were tested on the mapping population. These SSRs included 7 apple SSRs (CH02d08, CH02d12, CH03d02, CH04a12, CH04g07, CH04d10, and CH04d07; Liebhard *et al.* 2002) and 11 pear SSRs (NB111a, RLG1, NH024b, NB118a, NB105a, NH030a, NB135a, IPPN02, IPPN14, TsuENH044, and TsuENH102; Inoue *et al.* 2007, Nishitani *et al.* 2009, Yamamoto *et al.* 2002a, 200b, 2002c).

PCR analysis was performed in a total volume of 10 µL containing 5 µL of 2× GoTaq Colorless Master Mix (Promega), 0.4 µM each of forward and reverse primers, and 2 ng of genomic DNA. DNA was amplified in a GeneAmp PCR system 9700 (Life Technologies) programmed as follows: an initial denaturation step at 94°C for 5 min; 35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 1 min (extension); and a final extension at 72°C for 7 min. Amplified DNA fragments were separated using an Applied Biosystems 3130*xl* Genetic Analyzer (Life Technologies) with an internal size standard (GeneScan HD 400 ROX; Life Technologies). Data were collected and analyzed using the GeneMapper software version 5.0 (Life Technologies).

Development of novel SSR markers from apple genome sequences

SSR markers for fine mapping of the *Aki* gene were developed on the basis of synteny between the pear and apple genomes. The data sets for Malus × domestica Whole Genome v1.0 were downloaded from the Genome Database for Rosaceae (https://www.rosaceae.org/species/malus/malus_x_domestica/genome_v1.0). Apple contigs containing CH04h02 and CH03d02 regions were identified by BLASTN. The contigs between CH04h02 and CH03d02 were filtered out according to the GFF file. The identified contigs were searched for potential SSR sequences using the Tandem Repeats Finder software version 4.07b (Benson 1999) with the following alignment parameters: match = 2, mismatch = 11, and indel = 11. We detected di-nucleotide (at least 10 repeats) and tri-nucleotide (at least 6 repeats) SSR motifs. Based on these SSRs, 45 primer pairs (**Table 1**) were designed with the Primer3 software version 2.3.6 (Koressaar and Remm 2007, Untergasser *et al.* 2012) using the following parameters: length of 18–23 bp (optimum 20 bp), primer Tm of 56–63°C (optimum 60°C), a maximum Tm difference of 1°C, a primer GC content of 45–65% (optimum 55%), and a product size range of 90–360 bp. Novel apple SSR markers developed from the draft sequence of 'Golden Delicious' are indicated as Mdo.chr11. SSR analyses were performed as described above.

Linkage analysis and construction of genetic linkage group 11

Linkage group 11 of 'Kinchaku' was constructed using JoinMap 4.1 software (Van Ooijen 2006), and a pseudo-testcross strategy was applied to create genetic linkage maps (Grattapaglia and Sederoff 1994). An independence LOD score of 10.0 was used to define linkage groups. The regression mapping algorithm was selected for building linkage maps, and map distances were calculated according to Kosambi's mapping function (Kosambi 1943). The linkage map was drawn using MapChart 2.2 software (Voorrips 2002).

Homologous sequences around the Aki gene in the Chinese pear genome

We conducted BLASTN searches against a high-quality draft genome sequence of the diploid Chinese pear 'Dangshansuli' (Wu *et al.* 2013) (Pbr_v1.0; http://peargenome.njau.edu.cn/) to identify scaffolds with homology to the 1.5-cM region that includes the *Aki* locus of 'Kinchaku'. Apple genome sequences amplified by three primer pairs (Mdo.chr11.28, Mdo.chr11.30, and Mdo.chr11.34) were used as queries; an e-value cutoff of 1e-30 was used.

Results

Evaluation of black spot susceptibility or resistance

The parental cultivars and all F_1 progeny were evaluated for susceptibility or resistance to black spot. Identical results were obtained in all duplicate tests. The susceptible parent 'Kinchaku' showed severe symptoms, whereas the resistant parent 'Housui' showed no symptoms (**Fig. 1**).

Of 621 F_1 progeny of the 'Housui' × 'Kinchaku' cross, 309 showed no symptoms and 312 showed necrotic symptoms. Resistant and susceptible progeny could be clearly distinguished because no plantlets showed an intermediate response. The segregation ratio of resistant to susceptible plants fitted the expected ratio of 1:1 in the Chi-square test ($\chi^2 = 0.014$, P value = 0.904), in good accordance with previous reports, which used different combinations of susceptible and resistant parents (Kozaki 1973).

Linkage of Aki to SSR markers on linkage group 11

The SSR markers CH04h02 and CH03d02, developed from the apple genome sequence (Liebhard *et al.* 2002), show significant linkage to susceptibility genes *Ani* (in 'Osa Nijisseiki') and *Ana* (in 'Nansui') (Terakami *et al.* 2007). In the present study, CH03d02 showed scorable polymorphism, i.e., a heterozygous genotype in 'Kinchaku' and polymorphic band patterns between 'Housui' and 'Kinchaku',

Table 1. Characteristics of novel SSR markers developed from apple contigs

Marker name		Primer sequence (5′ to 3′)	Origin of apple contig	Contig start position (bp)	SSR start position in contig (bp)	Motif type[a]	Copy number[a]
Mdo.chr11.1	Forward:	CGAACTTCAGGTGAGTGGGT	MDC002629.387	2,513,421	3,294	GT	14.5
	Reverse:	AAGCATACATGTGCATCCCA					
Mdo.chr11.2	Forward:	ACACCGAGAGGACTCGAAGA	MDC002629.387	2,513,421	17,035	AT	16
	Reverse:	CTGTCTGCTTAGAGACGGGG					
Mdo.chr11.3	Forward:	TTCATGACCAACCCAGTTGA	MDC018137.214	3,057,173	7,237	GA	11
	Reverse:	AACAGACTGACGAACCGACC					
Mdo.chr11.4	Forward:	GTACCGGGTGCTTTTCGTTA	MDC011303.296	3,506,644	10,412	TC	10
	Reverse:	GTCTTGTGCTTGAGAGCGTG					
Mdo.chr11.5	Forward:	CCTTCATGAGGAACTCCATCC	MDC011303.296	3,506,644	20,684	TC	18
	Reverse:	TTGAGTTTGCAGCCATTGAG					
Mdo.chr11.6	Forward:	ATTGGGAGGGAAAAACAAGG	MDC010086.240	4,014,253	12,847	AG	11
	Reverse:	ACATTACTACGCCCTGCCAC					
Mdo.chr11.7	Forward:	TTGTTTGTCTGGGAAAAGGG	MDC019643.199	4,485,593	939	CAC	6.7
	Reverse:	GAATGTACGCACGGCTTTCT					
Mdo.chr11.8	Forward:	CACTCGCTATCCTCCTCCTG	MDC019643.199	4,485,593	11,432	CT	14
	Reverse:	GCAGAGTGGTGGGGTTTAGA					
Mdo.chr11.9	Forward:	TCTGTTTGAACCTCCATCCC	MDC020062.119	5,061,058	13,509	GA	13
	Reverse:	TTGTTGGCAACCTCACAAAC					
Mdo.chr11.10	Forward:	CAACCAACTGCTCAAGTCAA	MDC020062.119	5,061,058	15,569	AC	22.5
	Reverse:	TGCTGCTACTGTGAAGTTCG					
Mdo.chr11.11	Forward:	GGAAACCCTAACCATGCAAA	MDC020062.119	5,061,058	27,436	AG	22.5
	Reverse:	AGAGATACCAGAGGGGCGAT					
Mdo.chr11.12	Forward:	AGCCATGGCCACAGTTTAAT	MDC012329.239	5,445,700	23,490	ATG	7.7
	Reverse:	GGCCTGTTCGATATCTTTGC					
Mdo.chr11.13	Forward:	GCACAATTTCCCAAACAAGG	MDC019380.167	1,999,875	3,661	AT	23.5
	Reverse:	CGATATGCGTGCTAGGGAGT					
Mdo.chr11.14	Forward:	CCATGCATGACCTATTTCCC	MDC040598.17	2,138,383	10,758	AT	22
	Reverse:	TTCGATAAGTTGGCACGTCA					
Mdo.chr11.15	Forward:	CTGCGTTCGGTTAGATCACA	MDC005041.466	2,154,005	3,099	TA	11
	Reverse:	TTGCCTTATCGCCCATAATC					
Mdo.chr11.16	Forward:	CAAGAGGACTCGAAGATGCC	MDC015929.398	2,282,115	4,552	AT	12
	Reverse:	TCCGTTCCTCTCCGATACAC					
Mdo.chr11.17	Forward:	TACAAGGGTTTTGGGTCTCG	MDC004271.236	2,444,754	3,766	AG	14
	Reverse:	ATTGGTGGTGATTGGTTGGT					
Mdo.chr11.18	Forward:	TACCTAAAACCTGGAGGGGC	MDC020758.380	2,460,173	5,968	AT	10.5
	Reverse:	TACCACAAGGATTCCGGCTA					
Mdo.chr11.19	Forward:	CGCTAGAGGATGGGTTTTGC	MDC010399.168	2,470,302	1,066	TA	19
	Reverse:	GGCGTTGGAAGACCTATTGC					
Mdo.chr11.20	Forward:	GTGGAAACGGAAGTTGTGGT	MDC009710.295	2,554,186	3,571	TA	15.5
	Reverse:	TATCGGGTTCCAGACCACTC					
Mdo.chr11.21	Forward:	CACCAACGGTGTACGATGAG	MDC024524.27	2,613,603	20,758	AT	26
	Reverse:	CCACTAGATACGCACAAAACCA					
Mdo.chr11.22	Forward:	ATGATTTATGAAGGCAGCCG	MDC012704.279	2,659,409	14,275	TA	14.5
	Reverse:	GGTCCTCATCCGAAACTGAA					
Mdo.chr11.23	Forward:	GAGATATTGCCCTCCATTCG	MDC022706.249	2,698,057	22,759	AT	11
	Reverse:	CAAGACACTGTTGGATTCAGTC					
Mdo.chr11.24	Forward:	GTTCATGTTACGGATTGCAG	MDC002601.127	2,773,258	11,709	AT	12
	Reverse:	GAAAGCCTAGCCTCCTAGTCC					
Mdo.chr11.25	Forward:	CATGTTGTAAGCCCTCGGAT	MDC016602.78	2,811,403	13,177	AG	21.5
	Reverse:	ACTCCCACCAAGGAAGAGGT					
Mdo.chr11.26	Forward:	AGGGGATCCAATTCCTAACG	MDC018345.161	2,881,408	6,536	TC	10.5
	Reverse:	AAATCGTCACTGGAACTCCG					
Mdo.chr11.27	Forward:	TGACTTCAGCCTGCTAAACCT	MDC018345.160	2,897,025	6,262	AT	16
	Reverse:	TCACTCTCCCTTTTCATGCAC					
Mdo.chr11.28	Forward:	CAGATTCACCGAGTTGCAGA	MDC001444.132	2,910,449	4,945	AG	15
	Reverse:	AGGGTTTTGGTTGACAGTCG					
Mdo.chr11.29	Forward:	ACGCTCTTCCCAGCAAAATA	MDC003953.308	2,947,393	3,151	AG	15.5
	Reverse:	CCGTGGCGAAAATACAACTT					
Mdo.chr11.30	Forward:	TAACCGTTGCAAACCCTCTC	MDC017308.389	3,030,417	10,676	TC	11
	Reverse:	GATGGATGGAAACAAATGGG					
Mdo.chr11.31	Forward:	GCAAGGAACCAGCTGACAGT	MDC019519.287	3,049,141	607	TA	13.5
	Reverse:	CAGCACCGTCGTCTGATCTA					

Table 1. (continued)

Marker name		Primer sequence (5′ to 3′)	Origin of apple contig	Contig start position (bp)	SSR start position in contig (bp)	Motif type[a]	Copy number[a]
Mdo.chr11.32	Forward:	TCCTAAAGCAGCCACTCCTC	MDC027417.11	3,109,934	9,953	TAA	8
	Reverse:	GAGATGCCAACCAGGAAAAG					
Mdo.chr11.33	Forward:	GAGATCTCCTGCGTTTCTGG	MDC021160.220	3,150,224	6,672	GA	15.5
	Reverse:	TTCCCGCCCCTATCTCTATT					
Mdo.chr11.34	Forward:	AACAACCGAACCGATCTTTG	MDC021160.236	3,155,605	10,288	GA	15
	Reverse:	CGACGCGTACAATTCGTTAT					
Mdo.chr11.35	Forward:	TGATTTTTGGTGGAAGGCTC	MDC001844.336	3,186,360	14,311	TC	23
	Reverse:	GATCTCAATCAGGGACCCAA					
Mdo.chr11.36	Forward:	TGGACTCCAAACACCGAATAG	MDC016570.325	3,342,482	8,236	TA	19.5
	Reverse:	CCAAGAGGGACAAATTGGAA					
Mdo.chr11.37	Forward:	CTTTAGCGGTATGGCTTTGG	MDC005623.477	3,352,403	7,157	CT	14.5
	Reverse:	GAGGGCAGACAATTCACGAG					
Mdo.chr11.38	Forward:	TCCGAAGTGAGGCTACAAAA	MDC003776.129	3,542,189	22,300	TA	11.5
	Reverse:	CTTCTTCCAACTTGTTCGCC					
Mdo.chr11.39	Forward:	ATGTGGTGTCCTTTTGAGCC	MDC013005.433	3,579,304	30,338	AG	23.5
	Reverse:	GAATGTCTTGCTAGCCTCGG					
Mdo.chr11.40	Forward:	GGGTTTTCATGGGTGATGTT	MDC005218.335	3,632,162	13,823	AT	16
	Reverse:	TAAACCCGACCCGTTTACAG					
Mdo.chr11.41	Forward:	CCAACAAAGCACTCACATGG	MDC013149.562	3,661,752	8,716	AT	11.5
	Reverse:	TGTGCTCAAAAAGTGGATGC					
Mdo.chr11.42	Forward:	CGGTCCACTACTAGCCCTCA	MDC022698.364	3,695,978	5,597	TA	17
	Reverse:	GACCACATTGGTTTGAGAGTGA					
Mdo.chr11.43	Forward:	ATCGGTTACGTTTGCTTGGT	MDC010666.357	3,929,341	4,280	TC	16
	Reverse:	ATGAAGGAGTGGCTGCTTGT					
Mdo.chr11.44	Forward:	CCGGAAGGGTATTGTGAAAT	MDC003759.133	3,948,061	4,779	AT	24.5
	Reverse:	TGGCCAAGTATCAATGTGGA					
Mdo.chr11.45	Forward:	CCCTCGACAAGAATTGGGTA	MDC012710.225	3,984,326	12,533	GA	10.5
	Reverse:	CCTAACCGCCAGAAAAATCA					

[a] Motif type and copy number are estimated on apple genome sequences.

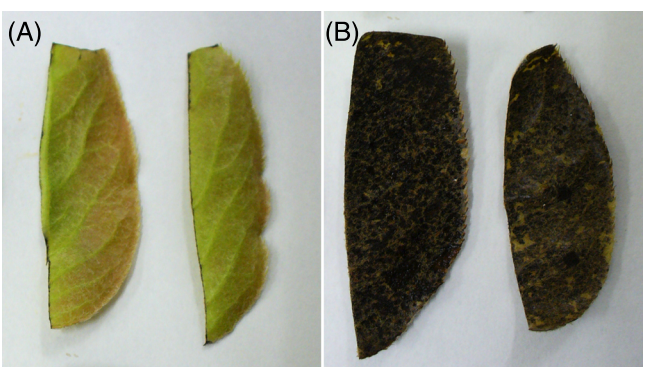

Fig. 1. Black spot disease symptoms of 'Housui' and 'Kinchaku' at 48 hours after inoculation with spore suspension of *A. alternata*. The resistant cultivar 'Housui' shows no visible disease symptoms (A), while necrotic lesions on leaves were observed in the susceptible cultivar 'Kinchaku' (B).

whereas CH04h02 was not polymorphic and therefore could not be scored. CH03d02 was significantly linked to *Aki* with a genetic distance of 17.6 cM (LOD score: 64.5). This result indicated that *Aki* was located at the top of linkage group 11, i.e., its position was very similar to those of *Ani* and *Ana*.

Map construction and fine mapping of Aki

Our results indicated that the susceptibility gene *Aki* was located between CH04h02 and CH03d02. To fine-map the *Aki* locus, we developed apple SSRs located between the two markers. In BLASTN searches, CH04h02 and CH03d02 showed high sequence similarity to apple genome contigs MDC010914.246 (e-value: 1e-116) and MDC005828.284 (1e-100), respectively. MDC010914.246 and MDC005828.284 are located on apple chromosome 11 (231,017–250,688 bp and 8,976,681–8,984,436 bp, respectively). We searched an approximately 8.7 Mb region on apple chromosome 11 (231,017–8,984,436 bp; 1,996 contigs) for di- and tri-nucleotide SSRs and designed 45 primer sets from those contigs (**Table 1**).

We also tested 18 SSR markers mapped previously on linkage groups 11 of pear or apple (Liebhard *et al.* 2002, Terakami *et al.* 2014) to establish linkage group 11 in 'Kinchaku'. Eight markers including CH03d02 were polymorphic in the mapping population and were mapped on linkage group 11 of 'Kinchaku'. Out of the 45 new apple SSR markers, 16 (36%) were polymorphic and were mapped on linkage group 11 of 'Kinchaku'; 14 markers (31%) showed no polymorphisms, 10 markers (22%) did not amplify bands of the expected size, and 5 markers (11%) were polymorphic but were not mapped on linkage group 11 of 'Kinchaku'.

Thus, the map of linkage group 11 of 'Kinchaku' consisted of 24 SSR markers and the black spot susceptibility gene (**Fig. 2A**). It spanned 73.1 cM and had an average marker

density of 2.9 cM per marker (**Supplemental Fig. 1**). *Aki* was located within a 1.5-cM genome region between the Mdo.chr11.28 and Mdo.chr11.34 markers (**Fig. 2B**). In the mapping population, three progeny were identified with recombination between Mdo.chr11.28 and *Aki* and six between Mdo.chr11.34 and *Aki*. No double recombination events were detected between Mdo.chr11.28 and Mdo.chr11.34. Mdo.chr11.30 co-segregated with *Aki* in all 621 F_1 plantlets. The segregation ratio was not distorted at any locus. The positions of novel apple SSR markers were well conserved in the Japanese pear (**Table 1**, **Fig. 2A**).

To verify that Mdo.chr11.30 was tightly linked to the genes for susceptibility to black spot disease, we carried out genotyping with Mdo.chr11.30 in 110 F_1 plantlets derived

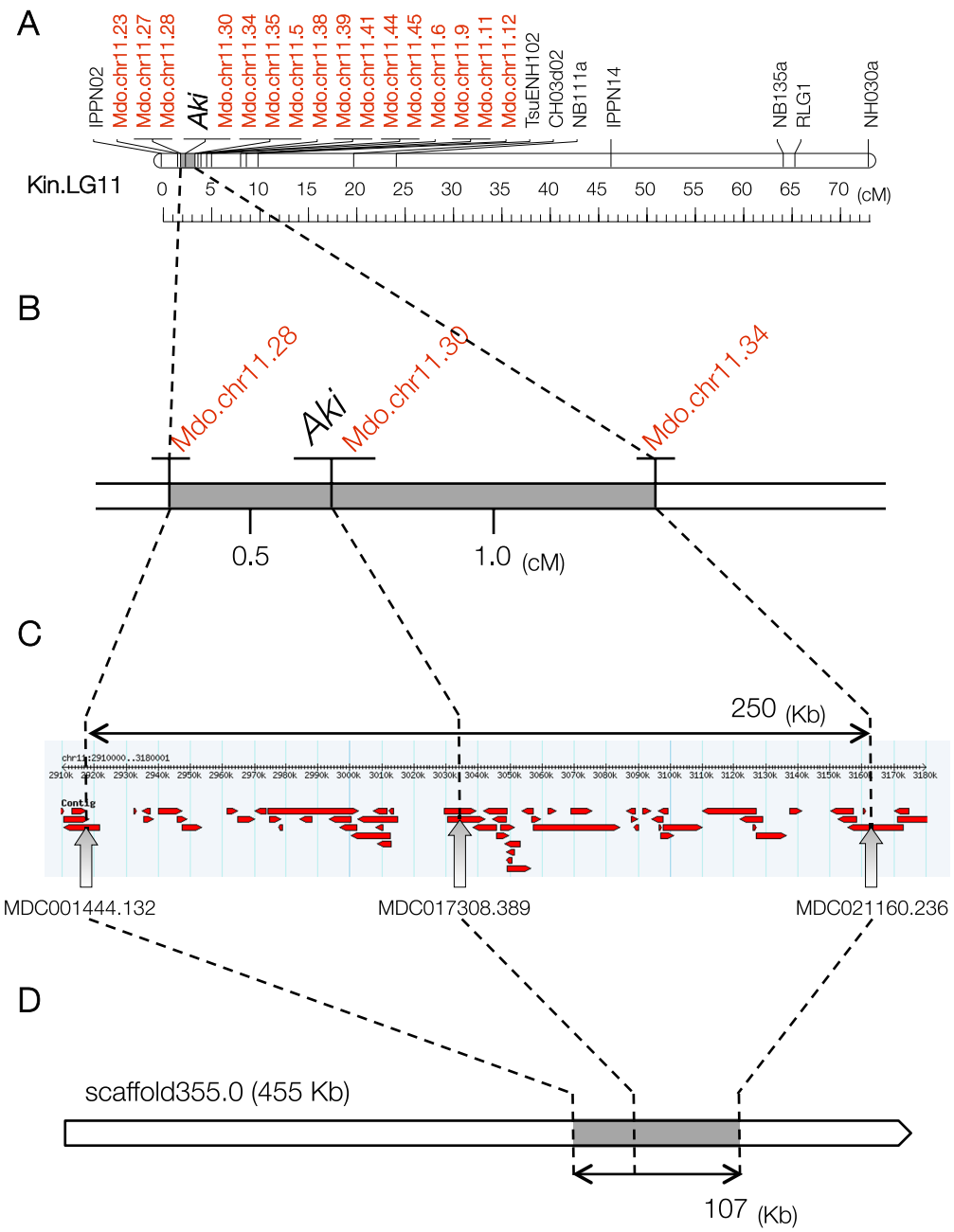

Fig. 2. Comparative map of Japanese pear, apple, and Chinese pear. A. Linkage group 11 in the Japanese pear 'Kinchaku'. *Aki*, the black spot susceptibility gene. Mdo.chr11, novel apple SSR markers developed from the 'Golden Delicious' draft genome sequence. The downside scale marks genetic distance (cM). B. Fine map of *Aki* and flanking regions. The numbers between markers indicate genetic distance (cM). C. Physical map of an apple genome segment (250 Kb) between Mdo.chr11.28 and Mdo.chr11.34 on chromosome 11 of 'Golden Delicious'. A screen snapshot of the Malus × domestica Whole Genome v1.0 view in GBrowse (https://www.rosaceae.org/gb/gbrowse/malus_x_domestica/). Red arrows indicate contigs. D. A scaffold of Chinese pear ('Dangshansuli') anchored to the region containing the *Aki* locus by using closely linked SSR markers (Mdo.chr11.28, Mdo.chr11.30, and Mdo.chr11.34). Block arrow indicates strand polarity.

Table 2. DNA sequences in the Chinese pear genome (Pbr_v1.0) homologous to novel apple SSR markers

SSR locus in the apple genome	Homologous scaffold in the Chinese pear genome	E-value	Alignment start position in Chinese pear scaffold (bp)	Alignment end position in Chinese pear scaffold (bp)
Mdo.chr11.28	scaffold289.0	1e-80	347,627	347,400
	scaffold355.0	2e-73	272,825	272,626
Mdo.chr11.30	scaffold355.0	4e-64	311,449	311,613
Mdo.chr11.34	scaffold355.0	1e-71	379,953	379,705
	scaffold153.0	1e-71	484,759	484,511

from a cross between 'Osa Nijisseiki' and 'Okusankichi', and found co-segregation of Mdo.chr11.30 with *Ani* (data not shown).

Aki was located between Mdo.chr11.28 and Mdo.chr11.34, which were developed from contigs MDC001444.132 and MDC021160.236, respectively (**Table 2**). The physical size of the region between these markers was approximately 250 Kb (2,915–3,165 Kb), including several gaps in the apple genome (**Fig. 2C**).

Scaffold containing Aki in the Chinese pear genome

Although a high-quality draft genome sequence of the diploid Chinese pear 'Dangshansuli' has been released and its 2103 scaffolds are available, their chromosomal positions are unknown. We surveyed Chinese pear scaffolds corresponding to apple genome sequences that include markers Mdo.chr11.28, Mdo.chr11.30, and Mdo.chr11.34.

Apple genome sequences amplified by the primer pairs corresponding to these three markers (**Table 1**) were used as queries in a BLASTN search against the Chinese pear genome (**Table 2**). Mdo.chr11.28 and Mdo.chr11.34 showed sequence similarity to multiple genome regions. The best hit for Mdo.chr11.28 was a 228-bp region in scaffold289.0 and the second best hit was in scaffold355.0 (272,626–272,825 bp; e-value: 2e-73). The latter scaffold also contained hits for Mdo.chr11.30 (a 165-bp region; 311,449–311,613 bp) and for Mdo.chr11.34 (a 249-bp region; 379,705–379,953 bp) (**Table 2**). These results indicated that the *Aki* gene is located in a 107-Kb region of scaffold355.0 (272,626–379,953 bp) of the Chinese pear genome (**Fig. 2D**).

Discussion

Using several anchored SSRs previously mapped in apple and pear (Silfverberg-Dilworth *et al.* 2006, Terakami *et al.* 2014), we constructed a genetic linkage map of linkage group 11 of the Japanese pear 'Kinchaku' that included the susceptibility gene *Aki*; the map consisted of 25 loci and spanned 73.1 cM (**Fig. 2A**, **Supplemental Fig. 1**). The position of *Aki* was identified at the top of linkage group 11. The marker CH03d02, which is linked to the susceptibility genes *Ani* of 'Osa Nijisseiki' and *Ana* of 'Nansui' (Terakami *et al.* 2007), was also significantly linked to *Aki* of 'Kinchaku'. The reference linkage maps of pear and apple were considered to be saturated (Silfverberg-Dilworth *et al.* 2006, Terakami *et al.* 2014). Comparison of the positions of anchored SSRs shows that the linkage map of 'Kinchaku' covers most of linkage group 11. Several RAPD markers linked to the susceptibility gene of 'Kinchaku' have been previously obtained and a genetic linkage map of 'Kinchaku' was constructed (120 loci, 18 linkage groups, 768 cM; Iketani *et al.* 2001). However, the linkage groups were not anchored to the reference linkage maps of pear or apple. The current study is the first to identify the position of *Aki*.

Several virulent and non-virulent isolates of *A. alternata* were reported, and strain No. 15A, used in this study, is an AK-toxin producer (Hayashi *et al.* 1990). All isolates of the pathogen that produce HSTs are pathogenic to the specific host; all isolates that fail to produce HSTs lack pathogenicity to the host plants (Tsuge *et al.* 2013). The range of pear cultivars sensitive to AK-toxin and spore suspension is the same as the host range of the pathogen (Kozaki 1974, Otani *et al.* 1985). The segregation ratio of resistant and susceptible progeny fitted the expected ratio of 1:1 in the Chi-square test. This result is in good agreement with a previous report that susceptibility of 'Kinchaku' to black spot is controlled by a single dominant gene (Kozaki 1973). In our previous study, we proposed different names (*Ani* and *Ana*) for the susceptibility genes in 'Osa Nijisseiki' and 'Nansui', respectively, because these genes are derived from different cultivars (Terakami *et al.* 2007). 'Osa Nijisseiki' is a self-compatible mutant of the native Japanese cultivar 'Nijisseiki' and is susceptible to black spot. 'Nansui' and 'Doitsu' are considered to carry the same susceptibility gene, *Ana*, because 'Nansui' is derived from the native Japanese cultivar 'Doitsu' (Terakami *et al.* 2007). In this study, we named the susceptibility gene of 'Kinchaku' as *Aki*; the parentage of this native Japanese pear cultivar has not been identified. All three susceptibility genes, *Aki*, *Ani*, and *Ana*, have been mapped at the top of linkage group 11, indicating that they are located very close to each other or have the same origin. Fine mapping of the *Aki* gene and determining nucleotide sequences flanking this gene were the starting point for a positional cloning of genes for susceptibility to black spot disease. The cloning of the susceptibility gene, *Aki*, represents the basis for further investigation of the susceptibility mechanism. It also takes a step toward identifying the homology and the functional relationships among susceptibility genes, *Aki*, *Ana*, and *Ani*.

In a genome-wide association study in the Japanese pear, Iwata *et al.* (2013) showed that the SSR marker CH04h02 was significantly associated with resistance to black spot. Although 76 Japanese pear varieties (31 modern elite cultivars, 19 old cultivars, 17 indigenous cultivars, and 9 breeding lines) were used in this study, 'Kinchaku' was not included. The 172-bp allele of CH04h02, which is linked in coupling phase with the susceptibility allele *Ani*, had the second-largest negative effect on resistance (Iwata *et al.* 2013). The *Aki* was located at the top of linkage group 11 and 'Kinchaku' has the 172-bp allele of CH04h02 support

the results of Iwata *et al.* (2013).

Several apple SSR markers have been successfully used in pears such as *P. pyrifolia*, *P. bretschneideri*, *P. ussuriensis*, *P. communis*, and *P. calleryana* (Yamamoto *et al.* 2001). Transferability of apple SSR information has been reported in some other species such as *Amelanchier canadensis*, *Cotoneaster dammeri*, *Cydonia oblonga*, *Sorbus domestica*, *Prunus armeniaca*, *P. persica*, and *P. salicina* (Liebhard *et al.* 2002). Similarly, SSR markers derived from pear and *Sorbus torminalis* were mapped on apple reference maps (Silfverberg-Dilworth *et al.* 2006). Pear reference linkage maps were constructed with apple SSR markers, and several apple SSRs were mapped on the same linkage groups in pear and apple (Terakami *et al.* 2009, Yamamoto *et al.* 2007). In addition, SSR loci were identified in similar regions of linkage groups, indicating collinear synteny between pear and apple in all 17 linkage groups.

Using a comparative genomic approach, we developed several apple SSR markers in the target region (the top of linkage group 11 in pear). Novel apple SSR markers developed from the draft sequence of 'Golden Delicious' are indicated as Mdo.chr11 (**Fig. 2A**). Of 45 novel apple SSR markers, 3 markers (Mdo.chr11.28, Mdo.chr11.30, and Mdo.chr11.34) were closely linked to the gene for susceptibility to black spot disease. The susceptibility gene, *Aki*, was located within a 1.5-cM genome region between Mdo.chr11.28 and Mdo.chr11.34, whereas Mdo.chr11.30 co-segregated with *Aki* in all 621 F_1 plantlets of the 'Housui' × 'Kinchaku' cross (**Fig. 2B**).

Alternaria blotch of apple, black spot of Japanese pear, and five other diseases are now known to be caused by *A. alternata* (Tsuge *et al.* 2013). Alternaria blotch, caused by the apple pathotype of *A. alternata*, is a destructive disease that can greatly reduce apple quality and yield. The degree of resistance or susceptibility of many cultivars has been previously reported (Abe *et al.* 2010, Saito and Takeda 1984). Saito and Takeda (1984) proposed that a single dominant gene, *Alt*, controls susceptibility to this disease and that the genotype of resistant cultivars is *alt*/*alt*. Using two populations of 57 each F_1 individuals derived from a cross between 'Starking Delicious' (susceptible, *Alt*/*alt*) and 'Jonathan' (resistant, *alt*/*alt*), Moriya *et al.* (2013) constructed linkage group 11 of 'Starking Delicious', which contained 12 markers spanning 22.1 cM, with an average of 1.8 cM between markers; *Alt* was located 6.7 cM from the top of linkage group 11 (Moriya *et al.* 2013). Alternaria blotch and black spot disease may have a common genetic basis in the two host species: host susceptibility is controlled by single dominant genes (*Alt* or *A*) located in similar positions at the top of linkage group 11 in both Japanese pear and apple.

A total of 54 contigs and several gaps were reported between MDC001444.132 and MDC021160.236, and 35 putative genes were predicted in the data sets for Malus × domestica Whole Genome v1.0. The susceptibility gene derived from 'Golden Delicious' is linked to the SSR marker CH05g07 at 5.6 cM (Li *et al.* 2011), but this marker was mapped on two linkage groups, 12 and 14 (Silfverberg-Dilworth *et al.* 2006). These results contradict the results of Moriya *et al.* (2013). This inconsistency may be caused by the use of different susceptible cultivars and inoculation methods (Moriya *et al.* 2013): Li *et al.* (2011) defined 'Golden Delicious' as susceptible, whereas other studies have found it to be resistant to Alternaria blotch (Abe *et al.* 2010, Saito and Takeda 1984). For these reasons, it is difficult to identify and characterize the gene for susceptibility to black spot disease using a comparative genomic approach on the basis of the published apple genome.

The Chinese pear genome size (527.0 Mb) is smaller than that of apple (742.3 Mb; Velasco *et al.* 2010), and the current version of the Chinese pear genome (Pbr_v1.0) is more informative for comparative genomics in the Japanese pear. The draft genome of the Chinese pear 'Dangshansuli', obtained using a combination of BAC-by-BAC and next-generation sequencing technologies, consists of 2103 scaffolds with an N50 of 540.8 Kb, and the total 512.0-Mb sequence corresponds to 97.1% of the estimated genome size (Wu *et al.* 2013). The draft genome of the European pear (Bartlett v1.0) has also been reported (Chagné *et al.* 2014), but this sequence is fragmented and consists of 142,083 scaffolds. Because the scaffolds of the Chinese pear genome are not anchored to chromosomes, we used the apple genome for synteny-based marker enrichment. Our results show that the susceptibility gene (*Aki*) is located within a 1.5-cM region between two apple SSR markers, Mdo.chr11.28 and Mdo.chr11.34 (**Fig. 2B**). We also surveyed the Chinese pear scaffold containing the *Aki* locus. A BLAST search against the Chinese pear genome revealed that all three markers showed high similarity to scaffold355.0 (**Table 2**). The physical size of the *Aki* locus defined by the three markers is 107 Kb (272,626–379,953 bp of scaffold355.0) in the Chinese pear genome (**Fig. 2D**).

Linkage maps were constructed to use for anchoring and orienting the scaffolds. A large number of single-nucleotide polymorphism (SNP) and SSR markers have been developed from the genome sequence of the Chinese pear, and several scaffolds have been anchored to 17 linkage groups (Chen *et al.* 2015, Wu *et al.* 2014). Wu *et al.* (2014) constructed a high-density linkage map of the Chinese pear using restriction-associated DNA sequencing technology, and a SNP marker developed from scaffold355.0 was mapped on linkage group 11. Their report supports our results. Five putative genes were predicted in the 107-Kb region, but those genes are not annotated in the Chinese pear genome. As information about the response of 'Dangshansuli' to black spot disease is no information, it is difficult to predict the candidate gene for the *Aki* locus in the Chinese pear genome.

Pear scab (caused by *Venturia nashicola*) is one of the most harmful diseases of pears, especially Japanese and Chinese pear species. *V. nashicola* is pathogenic only for Asian pears, and is not pathogenic for European pears (Bell *et al.* 1996, Ishii *et al.* 2002). None of the major commercial

Japanese pear cultivars are resistant to scab disease caused by *V. nashicola* (Bell *et al.* 1996, Ishii *et al.* 1992), but no scab symptoms were observed on the indigenous Japanese pear 'Kinchaku' (Abe and Kotobuki 1998, Ishii *et al.* 1992). Inheritance analysis indicated that this resistance is controlled by a single dominant gene (Abe and Kotobuki 1998). Six DNA markers, showing close linkages to a resistance gene, were identified, and the resistance gene was mapped in the central region of linkage group 1 (Terakami *et al.* 2006). Marker-assisted selection approach with regard to the pear scab resistance of 'Kinchaku' has been successfully tested (Gonai *et al.* 2009). However, 'Kinchaku' is susceptibility to black spot disease, while is resistance to pear scab. When 'Kinchaku' is used as the parent cultivar, about half of the progeny showed susceptibility to black spot disease. Molecular markers closely linked to the gene for susceptibility to black spot disease and the scab resistance gene will be useful for improving pear breeding by marker-assisted selection.

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