

A Simple Sequence Repeat Marker Linked to the Susceptibility of Apple to *Alternaria* Blotch Caused by *Alternaria alternata* Apple Pathotype

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ABSTRACT. *Alternaria alternata* apple pathotype (previously *A. mali*) causes alternaria blotch disease of apple (*Malus × domestica*), which may result in leaf spots and up to 70% premature leaf drop in serious cases. This disease is of worldwide importance but is most serious in eastern Asia (Japan, Korea, and China) and in parts of the United States. The excessive use of fungicides not only adds cost to apple growers, but also pollutes the environment. In this study, we characterized a 5-year F₁ population from a cross of a resistant cultivar (Huacui) and a susceptible cultivar (Golden Delicious) consisting of 110 individuals along with 14-year-old parent trees (10 each). A field evaluation of disease severity was conducted in 2008 and 2009 under the natural conditions in Liaoning, China (lat. 40°37' N, long. 120°44' E). Based on the field data, 110 F₁ plants were divided into five groups. Artificial inoculation was carried out both on the living trees and on the detached leaves in 2009 to ensure that *A. alternata* apple pathotype was the causative agent. Eighty primer pairs of simple sequence repeat (SSR) were screened against the four genomic DNA pools, respectively, from six highly susceptible F₁ plants, six most resistant F₁ plants, one tree of the seed parent, and the one tree of the pollen parent. One pair of primers (CH05g07) was shown to be linked to the DNA pools of susceptible F₁ and the parent tree, but not to the DNA pools of resistant F₁ and parent trees. This primer pair was then used to screen all individual 110 F₁ progenies and two parent trees. The differentiation of 103 individuals (97.3%) with the marker matched the field disease resistance rating. This marker was further screened with 20 cultivars with known susceptibility or resistance to *A. alternata* apple pathotype and its linkage to susceptibility was validated. These results suggest that this marker can be used in marker-assisted selection for resistance/susceptibility to alternaria blotch disease in apple.

Apple is the most cultivated fruit crop in the world, but many diseases reduce yield, quality, or lifespan of the trees, thereby restricting apple production (Jones and Aldwinckle, 1990).

Alternaria blotch can cause very serious damage in susceptible apple trees (Sawamura, 1990). The disease is caused by *Alternaria alternata* apple pathotype and was first reported in the United States (Roberts, 1924) but then was not considered a serious pathogen (Filajdic, 1995). Currently, the disease is of global significance and is one of the most serious fungal diseases affecting apple in Japan (Saito et al., 2001; Sawamura, 1990; Sekiguchi, 1976), Korea (Lee and Lee, 1972), the United States (Filajdic and Sutton, 1991), and China where nearly 50% of the world's apple is produced (Zhao et al., 2008).

Alternaria alternata apple pathotype can cause symptoms on leaves, young shoots, and fruit. Lesions appear first on the leaves in late spring or early summer as small round brownish

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Table 1. Assignment of each of the 110 F₁ ‘Golden Delicious’ × ‘Huacui’ apple trees based on field evaluations of alternaria blotch disease.

Value of grade	Diseased leaves (%) ^y	Evaluated in 2008 ^z		Evaluated in 2009 ^z	
		Total trees in category (no.)	Individual tree no.	Total trees in category (no.)	Individual tree no.
Highly resistant	10 or less	52	1, 2, 5, 6, 11, 13, 15, 17, 19, 21, 22, 24, 26, 29, 32, 33, 35, 36, 40, 41, 43, 45, 46, 48, 49, 50, 53, 54, 55, 58, 59, 60, 63, 64, 65, 68, 72, 73, 76, 81, 83, 84, 88, 89, 96, 98, 99, 100, 104, 108, 109, 110	35	1, 2, 5, 6, 11, 13, 15, 19, 21, 26, 29, 32, 35, 36, 41, 45, 48, 49, 50, 53, 58, 59, 60, 65, 68, 72, 73, 76, 81, 83, 89, 96, 98, 100, 104
Resistant	10–25	14	4, 9, 38, 51, 62, 66, 74, 90, 91, 92, 93, 94, 97, 106	28	4, 22, 24, 38, 40, 43, 46, 51, 54, 55, 62, 63, 64, 66, 74, 84, 88, 90, 91, 92, 93, 94, 97, 99, 106, 108, 109, 110
Susceptible	26–40	14	16, 20, 23, 27, 30, 34, 37, 42, 44, 47, 52, 67, 70, 101	16	9, 16, 17, 20, 23, 27, 30, 33, 34, 37, 42, 44, 47, 52, 67, 70
Moderately susceptible	41–50	10	75, 79, 85, 86, 87, 95, 102, 103, 105, 107	11	75, 79, 85, 86, 87, 95, 101, 103, 102, 105, 107
Highly susceptible	50 or greater	20	3, 7, 8, 10, 12, 14, 18, 25, 28, 31, 39, 56, 57, 61, 69, 71, 77, 78, 80, 82	20	3, 7, 8, 10, 12, 14, 18, 25, 28, 31, 39, 56, 57, 61, 69, 71, 77, 78, 80, 82

^zThe evaluations were conducted on 20 June in 2008 and 2009 when the disease was most severe. For each individual tree, 120 leaves were sampled.

^yThe overall rating of resistance or susceptibility for each tree was calculated by the percentage of diseased leaves accounted for the total surveyed leaves.

or blackish lesions, gradually enlarging, with a brownish purple border (Zhang et al., 2004). When becoming serious, the disease can result in up to 70% premature defoliation (Filajdic and Sutton, 1991). The fungus can overwinter as mycelium on dead leaves, in mechanically injured twigs, or in dormant buds. Primary infection takes place ≈1 month after petals fall. The disease spreads further by conidia with the help of wind, rain, or insects. The quality of *A. alternata* apple pathotype-infected apple can be reduced without commercial value. Nearly all major globally important cultivars, like Fuji, Red Delicious, Golden Delicious, and Starkrimson, are highly susceptible; and older cultivars, like Rails, have also shown increasing susceptibility in recent years (Zhao et al., 2001). Dickens and Cook (1995) found that isolates of “toxicogenic” *A. alternata* apple pathotype from U.S.-only infected leaves of cultivars Indo and Red Gold. Abe et al. (2010) did an evaluation of apple genotypes and *Malus* species for resistance to alternaria blotch and established a modified six-class disease scale for the rating system.

Although use of fungicides such as mancozeb, ziram, thiram, oxine-copper,

Table 2. Eighty primer pairs used in screening of polymorphic simple sequence repeat markers related to resistance of *Alternaria alternata* apple pathotype in apple.

Primer pair	Forward/reverse primer sequences	
	Forward	Reverse
CH01a07b	AACCCATGAAACACAATCCC	GGAACGATCCATAGGTGGTG
CH01F02	ACCACATTAGAGCAGTTGAGG	CTGGTTTGTTCCTCCAGC
CH01H10	TGCAAAGATAGGTAGATATATGCCA	AGGAGGGATTGTTTGTGCAC
CH02B03b	ATAAGGATACAAAACCTACACAG	GACATGTTTGGTTGAAAACCTG
CH02B10	CAAGGAAATCATCAAAGATTCAAG	CAAGTGGCTTCGGATAGTTG
COLa	AGGAGAAAAGCGTTTACCTG	CAAGTGGCTTCGGATAGTTG
CH01d09	GCCATCTGAACAGAATGTGC	CCCTTCATTCAACATTTCCAG
CH01f03b	GAGAAGCAAATGCAAAACCC	CTCCCCGGCTCCTATTCTAC
CH02b07	CCAGACAAGTCATCACAACACT	ATGTGCGATGTCGCTCTGTTG
CH02g09	TCAGACAGAAGAGGAACTGTAT	CAAACAAACCAGTACCGCAA
CH01c08	TCACTGTCCTTATCTTTACCAC	GCAGAAAAAGCACCCACC
CH01c11	AAATCCTAAAACACAAGCAAAACC	TGAACCAAGTCCCTCCACTCC
CH01d03	CCACTTGGCAATGACTCCTC	ACCTTACCGCAATGTGAAG
CH01d07	AAAATCCAGTTTTCCACCTC	AGTCGAAATCCCGAACAATC
CH01e09b	CCATCCAACACTGCCTTTCC	TTTGATGAACCCCTTCTTCC
CH01f07a	CCCTACACAGTTTCTCAACCC	CGTTTTTGGAGCGTAGGAAC
CH01f09(1)	ATGTACATCAAAGTGTGGATTG	AATCCAATTTTCAAGAACAGG
CH01g05	CATCAGTCTCTGCACCTGGAAA	GACAGAGTAAGCTAGGGCTAGGG
CH02b07	CCAGACAAGTCATCACAACACTC	ATGTCGATGTCGCTCTGTTG
CH02b11	ACCTTACCTGGTAGAGAGAGA	CACAACGCAGTTCGATCACT
CH02c02a	CTTCAAGTTCAGCATCAAGACAA	TAGGGCACACTTGCTGGTC
CH02c06(1)	TGACGAAATCCACTACTAATGCA	GATTGCGCGCTTTTTAACAT
CH02c09	TTATGTACCAACTTTGCTAACCTC	AGAAGCAGCAGAGGAGGATG
CH02e02	CTCATCAGTCTCACTGACTGTGTG	AGGGTCAGGGTCAGTCAGG
CH02e12	CCAACTTTTCTGCGGTAGTG	TGGGACCCATATGGTTGAATAC
CH02g04	TTTTACCTTTTACGTAAGTGGCG	AGGCAAACTCTGCAAGTCC
CH02g09	TCAGACAGAGGAACTGTATTTG	CAAACAAACCAGTACCGCAA
CH02h07	TGAGCTGACAAGTGAAAATGC	GCCGAACAATGTAAAGCTCG

continued next page

Table 2. Continued.

Primer pair	Forward/reverse primer sequences	
	Forward	Reverse
CH02h11b	GGGACGTAAACAGGTATTCTCTC	ATGGTTAGGCCAAGCACATC
CH03a03	GTGGTGGTAATGACGAGAACCT	AAGCAAAGTAGCCAACTGCAT
CH01h11	ACGCATCTCTCAGTTTGAAG	TTTATAGGCAGAGACCTTCAG
CH03a04	GACGCATAACTTCTCTCCACC	TCAAGGTGTGCTAGACAAGGAG
CH03a08	TTGGTTTGCTAGGAAAAGAAGG	AAGTTTATCGGGCCTACACG
CH03b01	ACAAGGTAACGTACAACCTCTCTC	GTCACAAAACCCGCCAGATG
CH03b06	GCATCCTTGAATGAGGTTCACT	CCAATCACCAAATCAATGTCAC
CH03b10	CCCTCCAAAATATCTCCTCCTC	CGTTGTCTGCTCATCATACTC
CH03c02	TCACTATTACGGGATCAAGCA	GTGCAGAGTCTTTGACAAGGC
CH03d02	AAACTTTCACTTTACCCACG	ACTACATTTTTAGATTTGTGCGTC
CH03d10	CTCCCTTACCAAAAACACCAA	GTGATTAAGAGAGTGATCGGGG
CH03d12	GCCCAGAAGCAATAAGTAAACC	ATTGCTCCATGCATAAAGGG
CH03g04	ATGTCCAATGTAGACACGCAAC	TTGAAGATGGCCTAACCTTGTT
CH03g06	ATGCCACAGCTTCTGTTTTTG	TCACAGAGAATCACAAGGTGGA
CH03g07	AATAAGCATTCAAAGCAATCCG	TTTTTCCAAATCGAGTTTCGTT
CH03h03	AAGAAATCGGATCCAAAACAAC	TCCCTCAAAGATTGCTCCTG
CH03h06	TTGTCCCTTTTTACGTCTTTCC	GTTATTGAGCAAGGCGGAGA
CH04c10	GGGTTAGGTTGTCTTCTCTCCT	GCTTCTCGGGTGAGTTTTTC
CH04d07	TGTCCTCCAATCTTAACCCG	CACACAGACGACACATTCACC
CH04e05	AGGCTAACAGAAATGTGGTTTG	ATGGCTCCTATTGCCATCAT
CH04e12b	CCTGAAATCTGCACAACCTACCA	GGTGGTGAAGAAGTAGACAGCC
CH04f06	GGCTCAGAGTACTTGCAGAGG	ATCCTTAAGCGCTCTCCACA
CH04f07	CAGATCATGAATGATTGAAA	GAAAATCACACCCTCAAACCAT
CH04f10	GTAATGGAAATACAGTTTCACAA	TTAAATGCTTGGTGTGTTTTGC
CH04g04	AGTGGCTGATGAGGATGAGG	GCTAGTTGCACCAAGTTCACA
CH04g10	CAAAGATGTGGTGTGAAGAGGA	GGAGGCAAAAAGAGTGAACCT
CH04g12	CACCTGATGGTGTCAACTTGT	CAACAAAATGTGATCGCCAC
CH05a02	GTTGCAAGAGTTGCATGTTAGC	TTTTGACCCATAAAAACCCAC
CH05a04	GAAGCGAATTTTGCACGAAT	GCTTTTGTTCATTGAATCCCC
CH05a05	TGTATCAGTGGTTTGCATGAAC	GCAACTCCCAACTCTTCTTTCT
CH05a09	TGATTTAGACGTCCACTTCACCT	TGATTGGATCATGGTGACTAGG
CH05b06	ACAAGCAAACCTAATACCACCG	GAGACTGGAAGAGTTGCAGAGG
CH05c02	TTAAACTGTCACCAAATCCACA	GCGAAGCTTTAGAGAGACATTCC
CH05c04	CCTTCGTTATCTTCTTGCATT	GAGCTTAAGAATAAGAGAAGGGG
CH05c06	ATTGGAACCTCTCCGATTGTGC	ATCAACAGTAGTGGTAGCCGGT
CH05c07	TGATGCATTAGGGCTTGTACTT	GGGATGCATTGCTAAATAGGAT
CH05d02	AAACTCCCTCACCTCACATCAC	AATAGTCCAATGGTGTGGATGG
CH05d08	TCATGGATGGGAAAAAGAGG	TGATTGCCACATGTCAGTGTT
CH05d11	CACACCCTGATATCCGGGAC	GAGAAGGTGCTACATTCCTCAA
CH05e03	CGAATATTTTCACTCTGACTGGG	CAAGTTGTTGACTGCTCCGAC
CH05e04	AAGGAGAAGACCGTGTGAAATC	CATGGATAAGGCATAGTCAGGA
CH05e05	TCCTAGCGATAGCTTGTGAGAG	GAAACCACCAAACCGTTACAAT
CH05e06	ACACGCACAGAGACAGAGACAT	GTTGAATAGCATCCCAAATGGT
CH05f04	GATGATGGTGTCTCTCGTTATT	TTATGTTGGGTAATGTCTTCCG
CH05f06	TTAGATCCGGTCACTCTCCACT	TGGAGGAAGACGAAGAAGAAAG
CH05g01	TTTCATTCAACTTCACCTCTC	CTCCTTCCGATTCTTCTATTTC
CH05g02	AGTGCAGCTTTCAGCTCAGATT	AGTCAGACACACCAAAAATCCCT
CH05g03	GCTTTGAATGGATACAGGAACC	CCTGTCTCATGGCATTGTTG
CH05g05	ATGGGTATTTGCCATTCTTGC	CCTGAAGCAAGGGAAGTCATAC
CH05g07	CCCAAGCAATATAGTGAATCTCAA	TTCATCTCCTGCTGCAAATAAC
CH05h05	ACAGTGCACCTCTACGCGG	GTGCAGTGATTAGCATTGCTGT

fungicide burden apple growers with considerable production cost, risk of fungicide residue in the environment, and with chemical residue on final fruit products. Alternatively, environment-friendly methods for chemical control of *A. alternata* apple pathotype have not been intensively studied in apple but are urgently needed (Jung, 2007). Saito et al. (2001) irradiated several cultivars with X- or γ -rays and produced mutants resistant to alternaria blotch disease as assayed in vitro by resistance to chemically synthesized AM-toxin I of *A. alternata*.

Breeding for disease resistance is one of the most effective alternatives, an alternative to fungicide use for apple disease management. Molecular markers are powerful tools in early selection for breeding disease-resistant apple; however, the effort has mainly focused on a few major diseases. Molecular markers have been identified to link to at least 10 different resistance genes for apple scab (Bus et al., 2002; Erdin et al., 2006; Gyax et al., 2004; Hemmat et al., 2003; Patocchi et al., 2005; Tartarini and Sansavini, 2002). Molecular markers and/or quantitative trait loci have also been found to link to powdery mildew [*Podosphaera leucotricha* (Calenge and Durel, 2006; Dunemann et al., 1999; Evans and James, 2003; James et al., 2004; Markussen et al., 1995)]. Others include fire blight [*Erwinia amylovora* (Janick et al., 1996; Khan et al., 2006)] and crown gall [*Rhizobium radiobacter* (Moriya et al., 2009)]. A random amplified polymorphic DNA (RAPD) molecular marker (S428-854) was linked to a gene resistant to alternaria leaf spot in apple (Zhao, 2008).

In this study, we conducted a field survey for 2 years on an F₁ population from the cross of 'Golden Delicious' (susceptible) and 'Huacui' (resistant Chinese cultivar derived from 'Golden Delicious' × 'Megumi') under natural conditions. We also conducted artificial inoculation in planta and in detached leaves to confirm the pathogenesis. We screened a set of SSR markers in the population, and one SSR marker was linked to

iminocadine, difenoconazole, tebuconazole, chlorothalonil, and polyoxin B against alternaria blotch has been reported and shown quite effective (Filajdic and Sutton, 1992; Gullino et al., 2000; Reuveni et al., 2002), multiple applications of

alternaria blotch susceptibility. This marker has also been verified in the entire F₁ population and 20 known susceptible and resistant cultivars, therefore validating the broad use of this SSR marker.

Materials and Methods

FIELD EVALUATION FOR *A. ALTERNATA* SUSCEPTIBILITY AND RESISTANCE. A 5-year segregating population consisting of 110 individuals derived from the cross of 'Golden Delicious' and 'Huacui,' along with 10 each of mature parent trees (14 years old), were evaluated for alternaria resistance and susceptibility. The trees were grown in an experimental orchard of the Institute of Pomology, Chinese Academy of Agricultural Sciences [Xingcheng, China (lat. 40°37' N, long. 120°44' E)]. Based on general disease severity in 2007, the evaluations were done in 2008 and 2009 on 20 June when the disease was most severe. No fungicide was applied and standard cultural practices were applied. The classification of disease rating was based on guidelines from the Institute for the Control of Agrochemicals (2000). For each individual tree, 120 leaves were sampled, and the overall rating of resistance or susceptibility for each tree was calculated by the percentage of diseased leaves accounting for the total surveyed leaves. Assignment of each tree for resistance or susceptibility is given as: highly resistant (10% or less disease leaves), resistant (11% to 25% diseased leaves), susceptible (26% to 40% diseased leaves), moderately susceptible (41% to 50% diseased leaves), or highly susceptible (50% or greater diseased leaves) (Table 1).

TO CONFIRM THE PATHOGENICITY OF *A. ALTERNATA* APPLE PATHOTYPE BY ARTIFICIAL INOCULATION. To confirm that the symptoms observed in the field were caused by *A. alternata* apple pathotype, fungus was isolated from the infected leaves of apple trees in the Fruit Plant Protection Research Center, Institute of Pomology (Xingcheng, China). Conidia were harvested from potato dextrose agar plates by adding 5 mL of double distilled water (ddH₂O) to each plate and gently rubbing the sporulating mycelial mat with a bent glass rod. Spore concentration was adjusted with a hemocytometer to 10⁵ spores/mL. The mature leaves showing no symptoms (10 from each tree) were brought to the laboratory on 10 June, washed briefly, and blotted dry with a paper towel. Spores were inoculated onto the back of leaves by even smearing. The inoculated leaves were placed on trays containing wet filter paper, covered with plastic bags to maintain high humidity, and held in a growth chamber (26 °C, 12 h of light per day) for 7 d. The disease symptoms were described and disease severity for each leaf was scored and averaged for 10 leaves from each tree.

Inoculation was also done in the tree on 25 June 2009. Shoots were inoculated by spraying conidial spores on the back of leaves on each shoot. After the inoculation, the shoots were maintained with high humidity by covering with wet plastic bags and removed the next morning. Control shoots were sprayed with distilled water. The rating of each leaf and the entire tree was the same as that used in the field disease evaluation.

ESTABLISHMENT OF RESISTANCE AND SUSCEPTIBILITY DNA POOLS. Based on the field evaluation of resistance and susceptibility in 2008 and 2009, six most resistant and six most susceptible individual plants in the F₁ population form resistant and susceptible DNA pools, respectively. One seed and one pollen parent tree were used to establish the parental DNA pools. Young leaves free of alternaria blotch were harvested from each individual tree and used immediately for DNA extraction or were frozen and stored at -70 °C until use.

DNA extraction followed the procedure of DNeasy Plant Mini Kit (Qiagen, Shanghai, China). The concentration was determined by spectrofluorometer (Shimadzu, Kyoto, Japan) at the absorbent wavelength of 260 and 280 nm. Gel electrophoresis (1.0% agarose) was used to determine the DNA purity with a single sharp band being considered pure. All individual DNA concentrations were adjusted to 100 ng·μL⁻¹ with ddH₂O. DNA (30 ng) of an equal amount from six individual resistant or susceptible plants was mixed to form the resistance DNA pool or susceptible DNA pool, respectively.

SCREENING OF POLYMORPHIC SIMPLE SEQUENCE REPEAT MARKERS. Eighty primer pairs (Table 2) were chosen from the 140 primer pairs, which were used by Liebhard et al. (2002) in seven cultivars and one breeding selection in the European D.A.R.E. project (Kellerhals et al., 2000). These 80 pairs were selected based on their high degree of polymorphism in other studies (Gygax et al., 2004; Khan et al., 2007; Silfverberg-Dilworth et al., 2006; Yamamoto et al., 2001; Yan, 2005). Primers were purchased from Shanghai Sangon Biological Engineering Technology and Services Co. (Shanghai, China). These 80 primer pairs were used in the first screening against the resistance and susceptibility DNA pools.

Each polymerase chain reaction (PCR) (20 μL) contained 2.0 μL of 10 × PCR buffer (Takara, Dalian, China), 2 μL of

Table 3. Apple cultivars that were verified the resistance of *Alternaria alternata* apple pathotype with the primer pair CH05g07.^z

Cultivar	Resistant (R) or susceptible (S)		Amplification with the primer pair CH05g07
	to <i>A. alternata</i> apple pathotype	Country of origin	
Akagi	R	Japan	No
Bayuesu	S	China	Yes
Belld de Boskoop	S	Netherlands	Yes
Cuihong	R	China	No
Dongguang	R	China	No
Fushuai	R	China	No
Huadiao	S	China	No
Huangguniang	S	China	Yes
Huashuai 1	S	China	Yes
Jinhong	R	China	No
Jonathan	S	USA	Yes
Kitanosach	R	Japan	No
Kogetsu	S	Japan	Yes
Liaofu	R	China	No
McIntosh	R	USA	No
Orin	S	Japan	Yes
Senshu	S	Japan	Yes
Tsugaru	R	Japan	No
Zaojinguan	R	China	No
Zipingguo	S	China	Yes

^zThe polymerase chain reactions were done in a manner similar to those reported in the Figure 3.

2.5 mmol·L⁻¹ each dNTPs (Takara), 0.2 μL of 5 U·μL⁻¹ Taq DNA polymerase (Takara), 1.6 μL of a 10 μmol·μL⁻¹ solution of each primer, 2 μL of 50 ng·μL⁻¹ genomic DNA, and 10.2 μL of ddH₂O. The PCR amplification was carried out in a T-gradient PCR (Whatman Biometra, Goettingen, Germany) with the following program: pre-denaturation at 94 °C for 4 min, 33 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 50 s, and extension at 72 °C for 1 min followed by a final extension at 72 °C for 10 min. The PCR products were resolved by 6% polyacrylamide gel electrophoresis. Before loading, the samples were denatured at 94 °C for 5 min. The gel was stained with silver nitrate (Bassam et al., 1991) to view the products, and the sizes of the bands were determined by comparison with the 100-bp DNA ladder (Takara).

VALIDATION OF THE CH05g07 SIMPLE SEQUENCE REPEAT MARKER. After the initial screening, the primer pair of CH05g07 was found to be polymorphic for distinguishing the resistance and susceptibility pools and seed and pollen parent DNA pools. This pair of primers was then further verified by testing all individual F₁ populations and 20 cultivars (Table 3) in which resistance or susceptibility to alternaria blotch was known (Lu and Jia, 1999). The DNA extraction, PCR reaction, and gel electrophoresis were same as described previously.

Results

Using the standard established in the field evaluation in 2007, the disease severity was fully evaluated in the field in 2008 and 2009. In 2008, 66 and 44 plants were classified to be, respectively, resistant (highly resistant and resistant) and susceptible (susceptible, moderately, and highly susceptible) in the 110 individuals of F₁ population of ‘Golden Delicious’ × ‘Huacui’. In 2009, 63 and 47 plants were scored to be resistant and susceptible, respectively. Among these plants, three trees (Trees 9, 17, and 33) were classified as resistant in 2008 but were rated as susceptible in 2009. The chi-square test ($\chi^2 = 4.4$) showed that the ratio of resistant trees to susceptible trees evaluated in 2008 did not conform to the 1:1 segregation ratios ($\chi^2_{0.05,1} = 3.84$), but the ratio in 2009 was in accordance with 1:1 segregation ($\chi^2 = 2.34$; $\chi^2_{0.05,1} = 3.84$).

To confirm the disease symptoms to be caused by the *A. alternata* apple pathotype, the pathogen was isolated and re-inoculated back to detached leaves and intact leaves in trees. Figure 1 shows the color features and the types of disease spots with two different inoculation procedures. When the leaves were inoculated directly in the trees, lesions colored lighter, and the sizes of lesions were smaller than those in the leaves that were inoculated in the illumination box. Despite the symptomatic differences, it was confirmed that *A. alternata* apple pathotype was the causative agent, which caused the same disease symptoms observed in natural conditions.

Among the 80 primer pairs, 14 produced polymorphic PCR bands between the resistance and the susceptibility DNA pools



Fig. 1. Symptom development after inoculation with *Alternaria alternata* apple pathotype in living apple trees in the orchard (A) and in detached leaves in an illumination box (B). Inoculation in the tree was done on 25 June 2009. Shoots were inoculated by spraying conidial spores on the back of leaves on each shoot. The shoots were then covered with wet plastic bags to maintain high humidity and removed the next morning. Control shoots were sprayed with distilled water. The detached leaves were inoculated by smearing conidial spores evenly onto the back of leaves and then placed on trays containing wet filter paper, covered with plastic bags, and held in a growth chamber (26 °C, 12 h of light per day) for 7 d. The disease symptoms were described and disease severity for each leaf was scored and averaged for 10 leaves from each tree. From left to right, a typical leaf rated as highly resistant, moderately resistant, resistant, susceptible, moderately susceptible, and highly susceptible, respectively.

with approximately five to 10 fragments being scored per primer pair (Fig. 2). However, only CH05g07 primer pair consistently generated a 163-bp band in the susceptibility DNA pool but not in the resistance DNA pool. This fragment was also amplified from the susceptible parent ‘Golden Delicious’ but not from the resistant parent ‘Huacui’ (Fig. 3). The additional weak band seen from Lane 3 in Figure 3 was estimated to be 180 bp and was therefore not the same band shown in the susceptible DNA pool. When CH05g07 was used to screen 110 F₁ individuals, 44 of 47 susceptible plants that were evaluated in the field or inoculation test in 2009 showed the CH05g07-163 fragment, and all 63 resistant individuals did not produce the band. The chi-square test ($\chi^2 = 3.28$) showed the segregation ratio was in accordance with 1:1 segregation ($\chi^2_{0.05,1} = 3.84$). The CH05g07 primers were further tested in 20 different apple cultivars. The resistant cultivars showed absence of the band and the susceptible cultivars showed the band.

Using Mapmaker/Exp software [Version 3.0b (Lander et al., 1987)], the CH05g07-163 SSR molecular marker was linked to the alternaria blotch-susceptible gene in apple; the distance of the marker to the susceptibility trait locus is ≈ 5.6 cM.

Discussion

Alternaria blotch is a serious apple disease worldwide, which could cause up to 70% premature defoliation. Although fungicide application is effective in managing the disease, it adds considerable cost to apple production and potential risks of polluting the environment. Breeding for resistance would be the

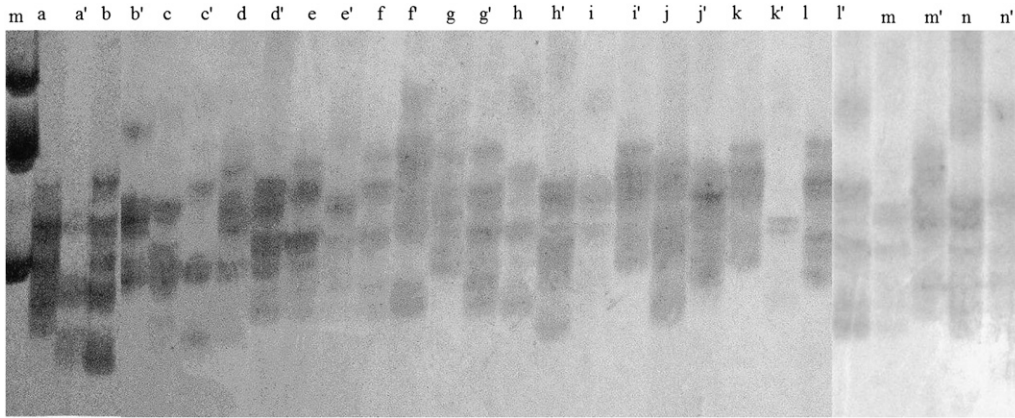


Fig. 2. Polymorphic simple sequence repeat patterns in the resistance and susceptibility DNA pools of F₁ 'Golden Delicious' × 'Huacui' apple trees amplified by 14 primer pairs: CH01c08 [Lane a = resistance DNA pool/Lane a' = susceptibility DNA pool (Lanes similar designations are used in this figure for primer pairs in Lanes b/b' to n/n')], CH05e05 (Lanes b/b'), CH02c02a (Lanes c/c'), CH02b07 (Lanes d/d'), CH05D11 (Lanes e/e'), CH02c09 (Lanes f/f'), CH04f06 (Lanes g/g'), CH03b06 (Lanes h/h'), CH04e05 (Lanes i/i'), CH01f07a (Lanes j/j'), CH01c08 (Lanes k/k'), CH05d11 (Lanes l/l'), CH05g07 (Lanes m/m'), CoLa (Lanes n/n').

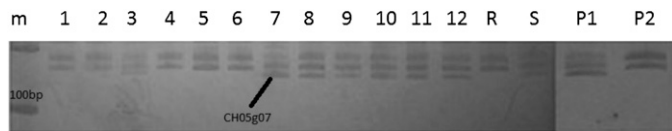


Fig. 3. Simple sequence repeat patterns showing the presence or absence of the CH05g07-163 fragment in apple, which can distinguish the resistance and susceptibility pools of *Alternaria alternata* apple pathotype and seed and pollen parent DNA pools; m = marker, 100-bp ladder, Lanes 1–6 = individuals in resistance bulk from the cross 'Golden Delicious' × 'Huacui', Lanes 7–12 = individuals in susceptible bulk from the cross 'Golden Delicious' × 'Huacui', R = bulk made of six resistant progenies, S = bulk made of six susceptible progenies, P1 = the susceptible parent ('Golden Delicious'), P2 = the resistant parent ('Huacui'). Arrows show the unique band linked to the susceptibility DNA pool and the parent tree, not to the resistance DNA pool or parent tree.

preferable strategy. Marker-assisted selection is a useful tool in accelerating the breeding program for disease resistance in apple (Bus et al., 2010, and references therein). In this study, one SSR marker that linked to the susceptibility to alternaria blotch was identified by using bulk segregation analysis. The usability of the bulked segregation analysis strongly relies on a precise resistance scoring (Koller, 1994). This is especially true for the rating of resistant plants, in which lack of disease symptoms can be the result of true genetic resistance or unsuccessful infection conditions. For this reason, the plants used in this study were evaluated for *A. alternata* apple pathotype resistance in 2 consecutive years, and only the extremely resistant and susceptible plants were used for establishing the resistance and susceptibility DNA pools. In this study, the field evaluations in 2008 and 2009 showed that only three trees (Trees 9, 17, and 33) were rated differently (classified as resistant in 2008 but susceptible in 2009); this difference is possibly the result of the weaker trees' vigor, which made the trees more susceptible. The conditions in orchards at the time of inoculation such as relative humidity, temperature, and tissue susceptibility could also affect rating the tree as resistant or susceptible. Some of the plants rated "highly resistant" in 2008 turned out to be "resistant" as a result of the greater rainfall in 2009 than that in 2008, which is more favorable for disease infection. The differences in symptom characters with inoculation on the field-grown trees

and on detached leaves also suggest that the environmental conditions affect the infection and symptom development. Nevertheless, the high consistency of disease rating allowed confident selection of candidate-resistant and susceptible trees for establishing DNA pools.

The availability of molecular markers linked to *A. alternata* apple pathotype susceptibility will be useful in future breeding programs without need for very time-consuming progeny tests. The CH05g07 SSR marker has been tested with 20 cultivars; the results showed that it was consistent with their field performance for alternaria blotch resistance or susceptibility. Previously, a S428-854 RAPD molecular marker linked to the alternaria blotch-resistant gene in apple was detected (Zhao, 2008). Therefore, it is now possible to use molecular markers to perform early screening of a breeding population for alternaria blotch resistance or susceptibility. With availability of many genomic resources, like BAC and YAC libraries and whole genome sequences to be released soon, it may be possible to link the marker further to the susceptibility or resistance gene and eventually lead to the isolation of the resistance or susceptibility gene.

Saito and Takeda (1984) suggested that the susceptibility to alternaria blotch in apple might be controlled by a single dominant gene, and most susceptible cultivars are heterozygous. The segregation ratios for alternaria blotch resistance/susceptibility based on the disease rating in the field in 2009 and on the CH05g07 SSR marker show the expected 1:1 segregation. This seems to support the assumption that the susceptibility to blotch is controlled by a dominant gene, and in the case of this study, the resistant parent, 'Huacui', used in generating the segregation population, is homozygous recessive, and the susceptible parent, 'Golden Delicious', is heterozygous dominant.

Literature Cited

- Abe, K., H. Iwanami, N. Kotoda, S. Moriya, and S. Takahashi (Sumiyoshi). 2010. Evaluation of apple genotypes and *Malus* species for resistance to alternaria blotch caused by *Alternaria alternata* apple pathotype using detached-leaf method. *Plant Breed.* 129:208–218.
- Bassam, B.J., G. Caetano-Anolle, and P.M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* 196:80–83.

- Bus, V., H. Bassett, D. Bowatte, D. Chagné, C. Ranatunga, D. Ulluwishewa, C. Wiedow, and S. Gardiner. 2010. Genome mapping of an apple scab, a powdery mildew and a woolly apple aphid resistance gene from open-pollinated mildew immune selection. *Tree Genet. Genomes* 6:477–487.
- Bus, V.G., P.A. Alspach, M.E. Hofstee, and L.R. Brewer. 2002. Genetic variability and preliminary heritability estimates of resistance to scab (*Venturia inaequalis*) in an apple genetics population. *N. Z. J. Crop Hort. Sci.* 30:83–92.
- Calenge, F. and C.E. Durel. 2006. Both stable and unstable QTLs for resistance to powdery mildew are detected in apple after four years of field assessments. *Mol. Breed.* 17:329–339.
- Dickens, J.S.W. and R.T.A. Cook. 1995. *Alternaria* pear black spot and apple blotch. Organisation Européenne et Méditerranéenne pour la Protection des Plantes/European and Mediterranean Plant Protection Organization (OEPP/EPPO) Bul 25.
- Dunemann, F., G. Bräcker, T. Markussen, and P. Roche. 1999. Identification of molecular markers for the major mildew resistance gene PI2 in apple. *Acta Hort.* 484:411–416.
- Erdin, N., S. Tartarini, G.A.L. Broggin, F. Gennari, S. Sansavini, C. Gessler, and A. Patocchi. 2006. Mapping of the apple scab-resistance gene *Vb*. *Genome* 49:1238–1245.
- Evans, K. and C. James. 2003. Identification of SCAR markers linked to PI-w mildew resistance in apple. *Theor. Appl. Genet.* 106:1178–1183.
- Filajdic, N. 1995. The influence of european red mites on intensity of *alternaria* blotch of apple and fruit quality and yield. *Plant Dis.* 79: 683–690.
- Filajdic, N. and T.B. Sutton. 1991. Identification and distribution of *Alternaria mali* on apples in North Carolina and susceptibility of different cultivars of apples to *alternaria* blotch. *Plant Dis.* 75:1045–1048.
- Filajdic, N. and T.B. Sutton. 1992. Chemical control of *alternaria* blotch of apples caused by *Alternaria mali*. *Plant Dis.* 76:126–130.
- Gullino, M.L., P. Leroux, and C.M. Smith. 2000. Uses and challenges of novel compounds for plant disease control. *Crop Prot.* 19:1–11.
- Gygax, M., L. Gianfranceschi, R. Liebhard, M. Kellerhals, C. Gessler, and A. Patocchi. 2004. Molecular markers linked to the apple scab resistance gene *Vbj* derived from *Malus baccata jackii*. *Theor. Appl. Genet.* 109:1702–1709.
- Hemmat, M., S.K. Brown, H.S. Aldwinckle, S.A. Mehlenbacher, and N.F. Weeden. 2003. Identification and mapping of markers for resistance to apple scab from ‘Antonovka’ and ‘Hansen’s baccata #2’. *Acta Hort.* 622:153–161.
- Institute for the Control of Agrochemicals. 2000. Guidelines for field trails of pesticide. Ministry of Agriculture GB/T 17980.124. Standards Press of China, Beijing, China.
- James, C.M., J.B. Clarke, and K.M. Evans. 2004. Identification of molecular markers linked to the mildew resistance gene PI-d in apple. *Theor. Appl. Genet.* 110:175–181.
- Janick, J., J.N. Cummins, S.K. Brown, and M. Hemmat. 1996. Apples, p. 1–77. In: Janick, J. and J. Moore (ed.). *Fruit breeding*. Vol. 1. Tree and tropical fruits. Wiley, New York, NY.
- Jones, A. and H. Aldwinckle. 1990. *Compendium of apple and pear diseases*. APS Press, St. Paul, MN.
- Jung, K.H. 2007. Growth inhibition effect of pyrroligneous acid on pathogenic fungus, *Alternaria mali*, the agent of *alternaria* blotch of apple. *Biotechnology* 12:318–322.
- Kellerhals, M., L. Gianfranceschi, N. Seglias, and C. Gessler. 2000. Marker-assisted selection in apple breeding. *Acta Hort.* 521:255–261.
- Khan, M.A., B. Duffy, C. Gessler, and A. Patocchi. 2006. QTL mapping of fire blight resistance in apple. *Mol. Breed.* 17:299–306.
- Khan, M.A., C.E. Durel, B. Duffy, D. Drouet, M. Kellerhals, C. Gessler, and A. Patocchi. 2007. Development of molecular markers linked to the ‘Fiesta’ linkage group 7 major QTL for fire blight resistance and their application for marker-assisted selection. *Genome* 50:568–577.
- Koller, B. 1994. DNA markers linked to *Malus floribunda* 821 scab resistance. *Plant Mol. Biol.* 26:597–602.
- Lander, E.S., P. Green, J. Abrahamson, A. Barlow, M.J. Daly, S.E. Lincoln, and L. Newburg. 1987. *Mapmaker: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations*. *Genomics* 1:174–181.
- Lee, D.H. and G.E. Lee. 1972. Studies on causal agents, overwintering of organisms and control of *alternaria* leaf spot of apple. *J. Korean Soc. Hort. Sci.* 11:41–47.
- Liebhard, R., L. Gianfranceschi, B. Koller, C.D. Ryder, R. Tarchini, E. Van De Weg, and C. Gessler. 2002. Development and characterization of 140 new microsatellites in apple (*Malus × domestica* Borkh.). *Mol. Breed.* 10:217–241.
- Lu, Q.-N. and D.-X. Jia. 1999. *Fruit trees of china-apple*. China Agriculture Press, Beijing, China [in Chinese].
- Markussen, T., J. Krüger, H. Schmidt, and F. Dunemann. 1995. Identification of PCR-based markers linked to the powdery-mildew-resistance gene PI (1) from *Malus robusta* in cultivated apple. *Plant Breed.* 114:530–534.
- Moriya, S., H. Iwanami, S. Takahashi, N. Kotoda, K. Suzuki, T. Yamamoto, and K. Abe. 2009. Genetic mapping of the crown gall resistance gene of the wild apple *Malus sieboldii*. *Tree Genet. Genomes* 6:195–203.
- Patocchi, A., M. Walser, S. Tartarini, G.A. Broggin, F. Gennari, S. Sansavini, and C. Gessler. 2005. Identification by genome scanning approach (GSA) of a microsatellite tightly associated with the apple scab resistance gene *Vm*. *Genome* 48:630–636.
- Reuveni, M., D. Sheglov, N. Sheglov, R. Ben-Arie, and D. Prusky. 2002. Sensitivity of red delicious apple fruit at various phenologic stages to infection by *Alternaria alternata* and moldy-core control. *Eur. J. Plant Pathol.* 108:421–427.
- Roberts, J.W. 1924. Morphological characters of *Alternaria mali* Roberts. *J. Agr. Res.* 27:699–708.
- Saito, A., N. Nakazawa, and M. Suzuki. 2001. Selection of mutants resistant to *alternaria* blotch from in vitro-cultured apple shoots irradiated with X- and [gamma]-rays. *J. Plant Physiol.* 158:391–400.
- Saito, K. and K. Takeda. 1984. Studies on breeding of apple. VIII genetic analysis of resistance to *alternaria* blotch (*Alternaria mali* Roberts) in apple. *Jpn. J. Breeding* 34:197–207 [in Japanese].
- Sawamura, K. 1990. *Alternaria* blotch, p. 24–25. In: Jones, A. and H. Aldwinckle (ed.). *Compendium of apple and pear diseases*. APS Press, St. Paul, MN.
- Sekiguchi, A. 1976. Studies on the *alternaria* leaf spot disease of apples caused by *Alternaria mali* Roberts. *Bul. Nagono Hort. Expt. Sta.* 12: 1–63.
- Silfverberg-Dilworth, E., C. Matasci, W. Van de Weg, M. Van Kaauwen, M. Walser, L. Kodde, V. Soglio, L. Gianfranceschi, C. Durel, F. Costa, T. Yamamoto, B. Koller, C. Gessler, and A. Patocchi. 2006. Microsatellite markers spanning the apple (*Malus × domestica* Borkh.) genome. *Tree Genet. Genomes* 2:202–224.
- Tartarini, S. and S. Sansavini. 2002. The use of molecular markers in pome fruit breeding. *Acta Hort.* 622:129–140.
- Yamamoto, T., T. Kimura, Y. Sawamura, K. Kotobuki, Y. Ban, T. Hayashi, and N. Matsuta. 2001. SSRs isolated from apple can identify polymorphism and genetic diversity in pear. *Theor. Appl. Genet.* 102:865–870.
- Yan, Z. 2005. Construction of an integrated map of rose with AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers. *Theor. Appl. Genet.* 110:766–777.
- Zhang, S., Y. Zhang, H. Liu, and J. Han. 2004. Isolation of *Alternaria mali* Roberts and its sensitivities to four fungicides. *J. Shanxi Agr. Univ. (Natural Sci. Ed.)* 24:382–384 [in Chinese].
- Zhao, J., L. Liu, X. Gong, and K. Wang. 2001. Resistance identification of some apple genus plants to leaf blotch. *Northern Fruits* 3:15 [in Chinese].
- Zhao, L. 2008. Detection of RAPD marker linked to the resistance gene to *alternaria* leaf spot in apples. Northwest A&F University Press, Yang Ling, China [in Chinese].
- Zhao, L., Z.Y. Zhao, Z.G. Dang, and X.C. Zhang. 2008. Heredity analysis on the hybrid of ‘Qinguan’ and ‘Fuji’ apple resistance to early defoliation disease. *Acta Agriculture Boreali-occidentalis Sinica* 17:197–201 [in Chinese].