Genetics and Molecular Marker Identification of a Resistance to Glomerella Leaf Spot in Apple

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Abstract

Apple Glomerella leaf spot (GLS) is a destructive fungal disease that damages apple leaves during the summer in China. Breeding new disease-resistant varieties is considered to be the best way of controlling GLS. A genetic study of resistance to Glomerella leaf spot (GLS) in apple was conducted by using four F\textsubscript{1} hybrid groups (‘Fuji’ × ‘Golden Delicious’, ‘Golden Delicious’ × ‘Fuji’, ‘Gala’ × ‘Fuji’, and ‘Fuji’ × ‘QF-2’) generated from two highly resistant varieties or selections, ‘Fuji’ and ‘QF-2’, and two highly susceptible varieties, ‘Golden Delicious’ and ‘Gala’. The results showed that the separation ratios of resistant plants to the susceptible ones in the four F\textsubscript{1} hybrid groups were statistically consistent with the theoretical ratios of 1:1, 1:1, 0:1, and 1:0. Comprehensive analysis enabled us to generate the following conclusions: GLS resistance in apple may be controlled by a single recessive gene. The genotype of the resistant plants was \textit{rr}, whereas the genotypes of the susceptible ones were \textit{RR} and \textit{Rr}. By using ‘Golden Delicious’ × ‘Fuji’ F\textsubscript{1} hybrid groups and the bulked segregation analysis (BSA) method, the marker S0506206-243bp associated with disease resistance character to GLS was identified through screening 500 SSR primers encompassing the entire apple genome with even coverage, and the genetic distance between the marker and the GLS resistance gene was 9.8 cM.

Keywords: apple; Glomerella leaf spot; inheritance of resistance; SSR marker

1. Introduction

Glomerella leaf spot (GLS) is a severe infectious disease caused by Glomerella cingulata that has recently affected apple production in China. It mainly targets apple leaves and causes black spots, ultimately leading to leaves drying out and falling off; it also infects fruits and causes necrotic lesions in the summer. This disease was first reported in Panama State, Brazil in 1988, when a new apple leaf spot disease was observed on the cultivars ‘Golden Delicious’ and ‘Gala’. The pathogenic bacterium, designated as \textit{G. cingulata} (Leite et al., 1988; González and Sutton, 1999; González, 2003), was the sexual form of Colletotrichum gloeosporioides, which was known as apple GLS. This disease was later detected in six apple-producing areas in Brazil from 1997 to 1999, and soon became the main disease affecting apples because of the extensive growth of the susceptible cultivar ‘Gala’ in Brazil (Crusius et al., 2002; Velho et al., 2013). In 1998, the disease was also observed in the United States (González and Sutton, 1999; González, 2003). Through further identification, Glomerella leaf spot in apples was considered to be caused by two pathogens, \textit{C. acutatum} and \textit{G. cingulata} (González et al., 2006), which respectively belonged to the \textit{C. acutatum} species complex and \textit{C. gloeosporioides} species complex (Wang et al., 2015b). In China, an apple leaf spot was noticed on ‘Gala’, ‘Golden Delicious’, and ‘Qinguan’ in August 2011 in Fengxian, Jiangsu Province, and then was confirmed as GLS caused by \textit{G. cingulata} (Song et al., 2012; Wang et al., 2012).

Further research by (Wang et al., 2015b) explicated that the pathogens causing this disease in China were \textit{C. fructicola} and \textit{C. aenigma}, which belonged to the \textit{C. gloeosporioides} species...
complex. No definite conclusion has been reached on whether the *C. acutatum* species complex exists in China.

From the field investigation of GLS in Laiyang, Shandong Province, Fengxian, Jiangsu Province, and Dangshan, Anhui Province, and the identification of indoor inoculation, we have determined that the resistance of apple to GLS significantly differed among various apple varieties. ‘Golden Delicious’, ‘Gala,’ and ‘Qinguan’ were apparently susceptible to GLS, whereas ‘Fuji’ and ‘Starkrimson’ were highly resistant. The result was consistent with the report of Becker et al. (2000) and Wang et al. (2015b).

Breeding and cultivating the disease-resistant varieties is considered to be one of the most effective measures to control the disease. Therefore, genetic studies and molecular marker screening of apple resistance to GLS are of great importance.

In the present study, two highly resistant varieties or selections, ‘Fuji’ and ‘QF-2’ (‘QF-2’ is the highly resistant selection obtained from a cross between ‘Qinguan’ and ‘Fuji’), and two highly susceptible varieties ‘Golden Delicious’ and ‘Gala’ were used to establish F1 hybrid populations. Resistance to GLS of 762 F1 individuals from the four populations were identified by artificial inoculation in the laboratory. The bulked segregant analysis (BSA) (Michelmore et al., 1991) method was used to identify SSR markers linked to the resistance gene. We aimed to reveal the pattern of inheritance of apple resistance to GLS and screen molecular markers that are closely linked to the resistant genes to improve breeding schemes for new GLS-resistant apple varieties.

### 2. Materials and methods

#### 2.1. Plant materials and inoculum

A total of 762 F1 individuals from the 4 segregation populations and 4 parents were used for artificial inoculation. The cross combinations were ‘Golden Delicious’ × ‘Fuji’ (207 F1 individuals), ‘Fuji’ × ‘Golden Delicious’ (95 F1 individuals), ‘Gala’ × ‘Fuji’ (262 F1 individuals), and ‘Fuji’ × ‘QF-2’ (198 F1 individuals). These seedlings were planted in the field of the Fruit Research Station of Qingdao Agricultural University (Jiaozhou, Shandong Province) in 2009.

The F1 progenies derived from the cross between ‘Golden Delicious’ × ‘Fuji’ were also used for the selection of SSR markers.

The pathogenic bacterium was *G. cingulata* (Wang et al., 2012), which was collected from the leaves of ‘Gala’ that showed fresh GLS lesions in September 2011 in an orchard located in Laixi, Shandong Province, China.

The leaves were incubated in moist Petri dishes at 25 °C for 3 days to promote pathogen sporulation. Single conidial isolates were obtained and transferred to potato dextrose agar (PDA) medium and allowed to grow at 25 °C for 2–3 days, then stored in the refrigerator at 5 °C. Before inoculation, the mycelia were transferred to fresh PDA medium and cultured at 25 °C until it covered about two-thirds of Petri dishes. Aerial mycelia were then scraped off by using an inoculating loop, and the rest of the mycelia in the Petri dishes were cultured at 25 °C for another 2–3 days. The newly formed orange conidia were collected using a sterilized inoculating loop and diluted with distilled water. The conidial suspensions to be used for inoculation were adjusted to a density of 10^6 conidia mL^{-1} using a hemocytometer.

#### 2.2. Sample collection and evaluation of GLS resistance

Four (two for inoculation identification and two as control) healthy shoots from every F1 individual were collected. Each shoot comprised four fully expanded leaves. The shoots were sterilized with 0.6% sodium hypochlorite, then washed with sterile distilled water. After a spray of conidial suspension of *G. cingulata*, the shoots were placed on a plate, which was then transferred into a plastic box with sterile water, then moved into an incubator without light at 25 °C. The GLS symptoms were evaluated 4 days later. The degree of resistance was classified as either resistant (R, no symptoms) or susceptible (S, small necrotic spots). The software SPSS13.0 was used for chi-square analysis.

In consideration of the fact that there is no effective prevention and control measure, this study did not conduct the field inoculation identification to prevent the spread of pathogens.

#### 2.3. DNA extraction and construction of resistant and sensitive pools

A total of 96 F1 individuals identified by artificial inoculation from the cross between ‘Golden Delicious’ and ‘Fuji’ were randomly selected for DNA extraction. Genomic DNA was extracted from 0.2 g of young leaves using the CTAB method (Tian et al., 2003). The DNA concentration of each sample was adjusted to 4 ng · μL^{-1}.

According to the phenotype of GLS resistance in vitro inoculation and the requirement of the BSA method, equal amounts of DNA extracted from 10 highly resistant plants and 10 highly susceptible plants were mixed to form two contrasting bulks.

#### 2.4. SSR marker development and linkage analysis

A total of 500 SSR markers evenly covering the 17 apple whole chromosomes were screened, including 300 primers previously published and 200 newly designed according to the genome sequence of ‘Golden Delicious’.

SSR-PCR reaction system was performed in a 15 μL volume, which included 2 μL of 4 ng · μL^{-1} of genomic DNA, 7.5 μL of the 1 × Master Mix, and 0.8 μL of 0.2 μmol · L^{-1} of each primer. PCR amplification was performed using the following conditions: pre-denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 40 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 8 min and then held at 4 °C. The amplified products were separated on 3.5% agarose gels.

The SSR marker genotypes of each of the 96 individuals were respectively assigned and recorded. The amplified band that was the same as the resistant bulk was labeled ‘A’, whereas that similar to the susceptible bulk was labeled ‘B’. The results of phenotype identification and the marker genotype data were used to calculate the genetic distance between the SSR marker and the resistance gene using Mapmaker 3.0.
3. Results

3.1. Assessment of GLS resistance in different apple varieties or strains

Identification of resistance of the five apple varieties or strains to GLS was conducted using the indoor inoculation test. ‘Fuji’ and ‘QF-2’ were determined to be highly resistant to GLS and did not show necrotic spots, whereas ‘Golden Delicious’, ‘Gala’, and ‘Qinguan’ were susceptible to GLS, with an average of >20 necrotic spots on each leaf (Table 1).

The results of the indoor inoculation test (Fig. 1) were consistent with the findings of the field investigation that was conducted in the apple production regions such as Dangshan, Anhui Province (Fig. 2).

The distinct difference in the resistance of various varieties (strains) demonstrated that genetic effects played a leading role in apple resistance to GLS. The present study has also verified that these varieties and strains could be utilized in genetic studies on apple resistance to GLS.

3.2. Phenotypic segregation ratio of hybrid populations derived from different combinations and resistance heredity

Table 2 shows that the phenotypic segregation ratios of the resistant individuals to the susceptible ones generated from

<table>
<thead>
<tr>
<th>Cross combination</th>
<th>Plants</th>
<th>Total plants</th>
<th>Resistant</th>
<th>Susceptible</th>
<th>Ratio of R to S</th>
<th>Expected ratio of R to S</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golden Delicious × Fuji</td>
<td>207</td>
<td>93</td>
<td>114</td>
<td></td>
<td>0.82:1</td>
<td>1:1</td>
<td>1.07</td>
<td>0.30</td>
</tr>
<tr>
<td>Fuji × Golden Delicious</td>
<td>95</td>
<td>40</td>
<td>55</td>
<td></td>
<td>0.73:1</td>
<td>1:1</td>
<td>1.20</td>
<td>0.27</td>
</tr>
<tr>
<td>Gala × Fuji</td>
<td>262</td>
<td>4</td>
<td>258</td>
<td></td>
<td>0.02:1</td>
<td>0:1</td>
<td>—</td>
<td>0.12</td>
</tr>
<tr>
<td>Fuji × QF-2</td>
<td>198</td>
<td>195</td>
<td>3</td>
<td></td>
<td>65:1</td>
<td>1:0</td>
<td>—</td>
<td>0.25</td>
</tr>
</tbody>
</table>
‘Golden Delicious’ × ‘Fuji’, ‘Fuji’ × ‘Golden Delicious’, ‘Gala’ × ‘Fuji’, and ‘Fuji’ × ‘QF-2’ were 0.82:1, 0.73:1, 0.02:1, and 65:1, respectively, which were in concordance with the theoretical ratios of 1:1, 1:1, 0:1, and 1:0 using the chi-square test. The results indicated that apple resistance to GLS was controlled by a single recessive gene. Therefore, the genotype of the resistant plant was designated as \( rr \), whereas that of the susceptible one was \( RR \) or \( Rr \). We thus speculated that the genotypes of the parental varieties used in different crosses using ‘Fuji’, ‘Golden Delicious’, ‘Gala’, and ‘QF-2’ were \( rr \), \( Rr \), \( RR \), and \( rr \), respectively. Similarly, the genotype of ‘Qinguan’ was determined to be \( Rr \) (Fig. 3).

3.3. Screening and verification of SSR markers linked to the GLS resistance gene

A total of 67 out of 500 pairs of SSR primers screened among the \( F_1 \) population of ‘Golden Delicious’ × ‘Fuji’ showed significant polymorphic bands between the resistant and susceptible DNA bulks. These 67 SSR markers were further screened among the two bulks and the two parents. Only 1 pair of primer (S0506206) clearly identified the two bulks and the two parents. The size of the amplified bands was 243 bp.

The genotypes of 96 \( F_1 \) individuals of ‘Golden Delicious’ × ‘Fuji’ were assessed using the primer S0506206. A total of 41 out of 44 susceptible plants generated a specific band (243 bp in size), whereas 48 out of 52 resistant ones did not show this fragment (Fig. 4).

The findings on phenotypic performance as tested by inoculation and the genotypes assessed by SSR marker S0506206 were used in linkage analysis by using the Mapmaker 3.0 software. SSR marker S0506206 was significantly correlated with the apple GLS resistance gene, and was separated by a genetic distance of approximately 9.8 cM.

4. Discussion

The occurrence of diseases is the result of the interaction among the pathogen, host, and environment. Therefore, the conditions of inoculation and the pathogenicity of the inoculant may influence host resistance. The inoculation method used in the present study was based on the protocol of Wang et al. (2015a), which ensured the performance of host plants responding to the inoculated pathogen. The pathogen that was isolated from the leaves of ‘Gala’ plants infested with GLS was also highly pathogenic to the ‘Golden Delicious’ and ‘Qinguan’ cultivars. These findings ensured the reliability of our methods for detection of GLS resistance.

Currently, most of the reports on apple GLS have focused on the causative factors, pathogenesis, host response, and disease control (Jonkers, 1973; Borsboom, 1974; Kender and Jonkers, 1975; González et al., 2006; Wang et al., 2012, 2015a; Leonarde and Marciel, 2013; Velho et al., 2013). On the other hand, genetic studies on GLS resistance are limited. The study of molecular markers linked to resistance genes has also not been reported.

In the present study, GLS resistance in apple was assessed in \( F_1 \) individuals of four hybrid groups. It was concluded that the GLS resistance gene in apple was controlled by a single recessive gene, which is in agreement with the conclusion of Dantas et al. (2009). The genotypes of ‘Fuji’, ‘Golden Delicious’, ‘Gala’, ‘QF-2’, and ‘Qinguan’ were \( rr \), \( Rr \), \( RR \), \( rr \), and \( Rr \), respectively. The identification of genotypes of apple varieties to improve selection is essential for breeding new varieties that are GLS-resistant. SSR markers are widely distributed across the ‘Golden Delicious’ genome (Velasco et al., 2010). Statistics show that there are approximately 163 426 SSRs occurring across 17 apple chromosomes (Guan et al., 2011). SSR markers have the advantage of being highly reproducible, highly reliable, easy to operate, and co-dominant (Goulão and Oliveria, 2001; Wang et al., 2005; Zhang et al., 2007; Gao et al., 2011). Thus, these have been extensively used in apple genetic linkage map construction, genetic
diversity detection, gene location, and marker-assisted selection. Here, we selected an SSR marker, S0506206, which has been linked to a GLS resistance gene and with a genetic distance of 9.8 cM. This marker may be utilized as a reliable pre-selection marker for the identification and breeding of resistant apple hybrid trees, although more SSR markers that are located closer to the GLS resistance gene should be identified to enhance the accuracy of pre-selection.

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