May 2016. Horticultural Plant Journal, 2 (3): 121–125.

Horticultural Plant Journal

Available online at www.sciencedirect.com
The journal's homepage: http://www.journals.elsevier.com/horticultural-plant-journal

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

LIU Yuanxia[a,b], LI Baohua[c], WANG Caihong[b], LIU Chunxiao[b], KONG Xianghua[b], ZHU Jun[b], and DAI Hongyi[b,*]

[a] *Horticulture & Landscape College, Hunan Agricultural University, Changsha 410128, China*
[b] *Qingdao Key Laboratory of Genetic Improvement and Breeding in Horticultural Plants, College of Horticulture, Qingdao Agricultural University, Qingdao, Shandong 266109, China*
[c] *College of Agronomy and Crop Protection, Qingdao Agricultural University, Qingdao, Shandong 266109, China*

Received 23 November 2015; Received in revised form 7 January 2016; Accepted 2 March 2016

Available online 14 June 2016

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_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_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 *rr*, whereas the genotypes of the susceptible ones were *RR* and *Rr*. By using 'Golden Delicious' × 'Fuji' F_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 *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, *C. acutatum* and *G. cingulata* (González et al., 2006), which respectively belonged to the *C. acutatum* species complex and *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 *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 *C. fructicola* and *C. aenigma*, which belonged to the *C. gloeosporioides* species

* Corresponding author. Tel.: +86 532 86080008.
E-mail address: hydai@qau.edu.cn

Peer review under responsibility of Chinese Society for Horticultural Science (CSHS) and Institute of Vegetables and Flowers (IVF), Chinese Academy of Agricultural Sciences (CAAS)

The Chinese version of this paper is published in Acta Horticulturae Sinica. DOI:10.16420/j.issn.0513-353x.2015-0288

http://dx.doi.org/10.1016/j.hpj.2016.06.002

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 F_1 hybrid populations. Resistance to GLS of 762 F_1 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 F_1 individuals from the 4 segregation populations and 4 parents were used for artificial inoculation. The cross combinations were 'Golden Delicious' × 'Fuji' (207 F_1 individuals), 'Fuji' × 'Golden Delicious' (95 F_1 individuals), 'Gala' × 'Fuji' (262 F_1 individuals), and 'Fuji' × 'QF-2' (198 F_1 individuals). These seedlings were planted in the field of the Fruit Research Station of Qingdao Agricultural University (Jiaozhou, Shandong Province) in 2009.

The F_1 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 10^4 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 F_1 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 F_1 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.