Front. Agric. China 2011, 5(4): 430–436
DOI 10.1007/s11703-011-1107-1

RESEARCH ARTICLE

Identification and molecular tagging of two *Arabidopsis* resistance genes to *Botrytis cinerea*

Jihong XING*[1 *], Qiaoyun WENG*[2 *], Helong SI[1], Jianmin HAN[1], Jingao DONG (✉)[1]

[1] *Molecular Plant Pathology Lab, College of Life Science, Agricultural University of Hebei, Baoding 071001, China*
[2] *Department of Agricultural and Forest Technology, Hebei North University, Zhangjiakou 075131, China*

© Higher Education Press and Springer-Verlag Berlin Heidelberg 2011

Abstract **To map *Arabidopsis* resistance genes to *Botrytis cinerea*, *Arabidopsis* Col-0 ecotype resistant to *B. cinerea* BC18 isolate and *Arabidopsis* L*er* ecotype susceptible to *B. cinerea* BC18 isolate were crossed. According to the resistant responses of the F_1, BC_1 and F_2 populations to *B. cinerea*, we identified two genes, named *BC1* and *BC2*, responsible for the resistance of *Arabidopsis* L*er* ecotype to *B. cinerea*. Through the method of map-based cloning, *BC1* was linked to DNA markers CCR1 and DHS1 on the fourth chromosome of *Arabidopsis* with genetic distances of 1.2 cM and 1.6 cM for CCR1 and DHS1, respectively, and *BC2* was linked to DNA markers CA72/NGA151 and NGA106 on the fifth chromosome with genetic distances of 1.4 cM and 2.4 cM for CA72/NGA151 and NGA106, respectively. Our results are beneficial for chromosome walking so that we can obtain the whole gene sequences, which will facilitate the understanding of their roles and manners of resistance to *B. cinerea*.**

Keywords *Arabidopsis thaliana*, *Botrytis cinerea*, resistant genes, linkage analysis, gene mapping

Introduction

Botrytis cinerea, an important plant pathogenic fungus, causes a severe disease in a wide range of plant species both in the field and in postharvest situations and results in significant economic losses. The molecular and cellular mechanisms involved in plant resistance to *B. cinerea* and their genetic control have been poorly understood up to now. Having a small genomic DNA, short growth cycle and the completion of sequencing, *Arabidopsis thaliana* has been widely used as a model organism for researching cellular and molecular interactions between fungi and plants (The Arabidopsis Genome Initiative, 2000). In 2003, Mengiste et al. (2003) identified one *Botrytis*-susceptible loci (*BOS1*) encoding an R2R3MYB transcription factor that was required for biotic and abiotic stress responses in *Arabidopsis*. Ferrari et al. (2003) found that salicylic acid and camalexin were involved in the local resistance of *Arabidopsis* to *B. cinerea* and needed genes *EDS4* and *PAD2* to be turned on but genes *SID2*, *EDS5* or *PAD4* to be turned off. Veronese et al. (2004) identified three other *Arabidopsis*-susceptible genes, *BOS2*, *BOS3* and *BOS4*, which were necessary for *B. cinerea* infection. Two years later, they identified a membrane-anchored *Botrytis*-induced kinase 1 which played distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens (Veronese et al., 2006). Expression profiling and mutant analysis of *ZFAR1* and *WRKY70* showed an increase in *Arabidopsis* resistance to *B. cinerea* (AbuQamar et al., 2006). Dhawan et al. (2009) showed that *Arabidopsis HUB1* (HISTONE MONOUBIQUITINATION1) was a regulatory component of the plant defense from *B. cinerea*.

Previously, we examined the responses of 11 different *Arabidopsis* ecotypes and genotypes to the infection with 21 *B. cinerea* isolates. We found that various *Arabidopsis* ecotypes infected by the same *B. cinerea* isolate showed different symptoms. Among them, Col-0 ecotype appeared immune to the *B. cinerea* BC18 isolate infection, while L*er* ecotype exhibited susceptible symptoms (Xing et al., 2010). In this study, the resistant Col-0 ecotype and susceptible L*er* ecotype were crossed. On the basis of the responses of the F_1 and F_2 populations, we identified two genes mediating the resistance of *Arabidopsis* against infection from *B. cinerea*.

Received December 24, 2010; accepted January 10, 2011
Correspondence: Jingao DONG
E-mail: shmdjg@hebau.edu.cn

[*] They contributed equally to this work.

Through the method of map-based cloning, two disease-resistant genes were located on the fourth and fifth chromosome of *Arabidopsis*.

Materials and methods

Arabidopsis thaliana ecotypes and *B. cinerea* isolate BC18

Arabidopsis ecotypes Col-0 and L*er* were kindly provided by Dr Xi (Donald Danforth Plant Science Center, USA). Ecotype Col-0 appeared immune to the *B. cinerea* isolate BC18, but ecotype L*er* appeared susceptible to it. The *B. cinerea* isolate BC18 was provided by the Molecular Plant Pathology Laboratory, Agricultural University of Hebei, China.

Primers of SSLP (simple sequence length polymorphism)

The sequences of primers were obtained from http://www.arabidopsis.org/home.html. We selected 3 to 4 molecular primers that were distributed symmetrically on each chromosome (Table1). Primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.

Construction of mapping population and disease assay

Crosses between ecotypes of Col-0 (male parent) and L*er* (female parent) were performed, and the F_1 plants were selfed independently. F_1 plants, BC_1 and F_2 segregating population were used as the mapping population and the inheritance of their resistance to *B. cinerea* was documented. To infect plants, a 5 µL spore suspension (4×10^5–8×10^5 spores/mL) of *B. cinerea* isolate BC18 was dropped on individual leaves of 4-week-old plants. After inoculation, plants were kept under a transparent cover to maintain 100% relative humidity (RH) and kept in the dark for 24 h before being transferred to a growth chamber in the temperature range of 18°C to 22°C and a photoperiodic regime of 16 h light and 8 h dark. Disease course was scored between 1 and 5 d after inoculation. Susceptible plants were characterized by extensive tissue damage at an early stage after *B. cinerea* infection compared with resistant plants.

Selection of codominant primers

Genomic DNA was isolated from the leaves of individual plants using a modified CTAB procedure (Stewart and Via, 1993). The procedure of SSLP was taken from a CSHL course with an appropriate modification. The reaction system (25 µL) included 10 × PCR reaction buffer 2.5 µL, dNTP Mix (10 mmol/L) 2.5 µL, positive and negative primers (20 µmol/L) 0.5 µL respectively, template DNA 1.0 µL (DNA quantity: 500 ng/µL), *Taq* DNA polymerase 0.5 µL (enzyme quantity: 5 U/µL). The program for amplification was as follows: 95°C denature for 30 s; 55°C anneal for 30 s; 72°C extend for 1 min; and for 50 cycles. PCR product was examined by 4% agarose gel electrophoresis. The electricity parameters were 1–3 h, voltage 80–120 V, and 1 × TAE buffer solution.

Construction of resistant and susceptible pools of F_2 segregation population

The DNA of 10 resistant and 10 susceptible plants from the F_2

Table 1 SSLP markers used in this study

Markers	Chr.	Position	Col/L*er*	Primer sequence (5′–3′)
NGA692	1	119.25 cM	S110/90	TTTAGAGAGAGAGAGCGCGG AGCGTTTAGCTCAACCCTAGG
NGA63	1	11.48 cM	S111/89	AACCAAGGCACAGAAGCG ACCCAAGTGATCGCCACC
NGA280	1	83.83 cM	S105/85	CTGATCTCACGGACAATAGTGC GGCTCCATAAAAAGTGCACC
NGA392	1	41.64 cM	S170/162	TTGAATAATTTGTAGCCATG GGTGTTAAATGCGGTGTTC
CIW3	2	30 cM	S230/200	GAAACTCAATGAAATCCACTT TGAACTTGTTGTGAGCTTTGA
AthBIO2b	2	76.11 cM	S140/209	TGACCTCCTCCTCCATGGAG TTAACAGAAACCCAAAGCTTTC
CZSOD2	2	56.94 cM	S183/187	GCATTACTCCGGTGTCGTC GAATCTCAATATGTGTCAAC
NGA162	3	20.56 cM	S107/89	CATGCAATTTGCATCTGAGG CTCTGTCACTCTTTTCCTCTGG
NGA6	3	86.41 cM	S143/123	TGGATTTCTTCCTCTCTTCAC ATGGAGAAGCTTACACTGATC
GAPAB	3	43.77 cM	S142/150	CACCATGGCTTCGGTTACTT TCCTGAGAATTCAGTGAAACCC
NGA172	3	6.91 cM	S162/136	AGCTGCTTCCTTATAGCGTCC CATCCGAATGCCATTGTTC
NGA1139	4	83.41 cM	S114/118	TAGCCGGATGAGTTGGTACC TTTTTCCTTGTGTTGCATTCC
NGA1107	4	104.73 cM	S150/140	CGACGAATCGACAGAATTAGG GCGAAAAAACAAAAAAATCCA
NGA8	4	25.56 cM	S154/198	GAGGGCAAATCTTTATTTCGG TGGCTTTCGTTTATAAACATCC
NGA129	5	105.41 cM	S177/179	TCAGGAGGAACTAAAGTGAGGG CACACTGAAGATGGTCTTGAGG
CDPK9	5	44.55 cM	S106/104	GAAACTGACTTGGAGAAGGCA TCAATCATTGTCCAAAACTTGG
NGA76	5	68.4 cM	S220/300	GGAGAAAATGTCACTCTCCACC AGGCATGGGAGACATTTACG
NGA151	5	29.62 cM	S150/120	GTTTTGGGAAGTTTTGCTGG CAGTCTAAAAGCGAGAGTATGATG

segregation population were mixed respectively to form a resistant gene pool and a susceptible gene pool for bulk segregation analysis (Michelmore et al., 1991).

Molecular tagging of resistance genes to *B. cinerea*

Codominant primers were selected to analyze the pooled DNA of the F_2 segregation population for the initial linkage test. The SSLP primers that could distinguish between the resistant gene pool and the susceptible gene pool were linked with markers (Lukowitz et al., 2000). Once the linkage between the resistant gene and genetic markers was established, 144 individual F_2 resistant plants were further analyzed by PCR with at least two flanking markers to score the distance between the resistant gene and the corresponding markers.

The genetic distances (centimorgan, cM) between the resistant gene and markers were determined using MAPMAKER/EXP 3.0 (Lander et al., 1987) based on the segregation data of the plant resistance and the molecular markers of the F_2 mapping population, LOD $\geqslant$ 3.0. Genetic mapping was carried out using the MapChart software.

Results

Morphologic observation of hybrid plant

The morphologic observation of 1493 hybrid plants of the cross between Col-0 (male parent) and L*er* (female parent) indicated that 520 hybrid plants were similar to the female parent (L*er*), while 973 hybrid plants with light leaf color and long leafstalk were similar in character to the male parent (Fig. 1).

Selection of codominant primers

Taking the individual genomic DNA of the parents (Col-0 and L*er*) and 10 hybrid plants which were different from the female parent as templates, PCR was performed to select codominat SSLP primers that could identify hybrid plants (Fig. 2). Primers distributed on the first and second chromosomes could not distinguish parents and hybrid plants. These primers were unlinked to the cross site. The primers, including NGA162, NGA6, NGA172, NGA8, NGA1107, NGA151 and NGA76, could identify the hybrid plants. NGA162, NGA6 and NGA172 were distributed on the third chromosome, NGA8 and NGA1107 were distributed on the fourth chromosome, NGA151 and NGA76 were distributed on the fifth chromosome. PCR amplification results using the primers above showed that hybrid plants displayed characteristic bands of male and female parents. These primers were codominant primers which could identify parents and hybrid plants.

Identification of hybrid plants

According to the morphological identification, 120 of the 973 hybrid plants that were different from the female parent were selected. Taking individual genomic DNA of the hybrid plants as template, PCR amplification was performed using codominant primers NGA172 and NGA8. The real hybrid plant has two bands, which both male and female parents have, whereas non-hybrid plants has only one band which belongs to either the female or the male parent. Plants with other bands also were non-hybrid plants (Fig. 3). Through the identification of SSLP molecular markers, 119 of the 120 plants displayed two characteristic bands of male and female parents simultaneously and were considered real hybrid plants.

Resistance identification of F_1, F_2 and BC_1 progenies

After being inoculated with *B. cinerea* isolate BC18, 944 of 975 F_1 hybrid individuals from the cross Col-0 × L*er* were found resistant, while the other individuals showed susceptibility to *B. cinerea*. Among the 3270 F_2 individuals, 2631 displayed resistance and 639 individuals displayed susceptibility, and the ratio between resistant and susceptible plants fit

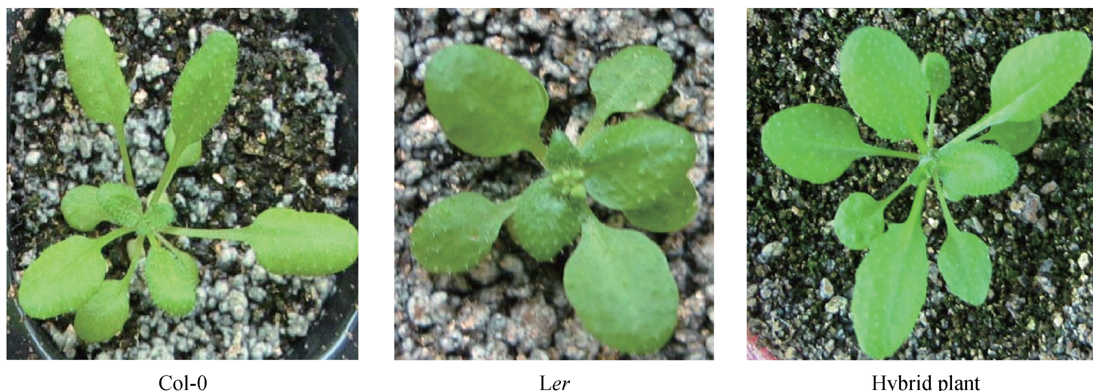

Figure 1 Morphologic comparison between hybrid plant and parents.

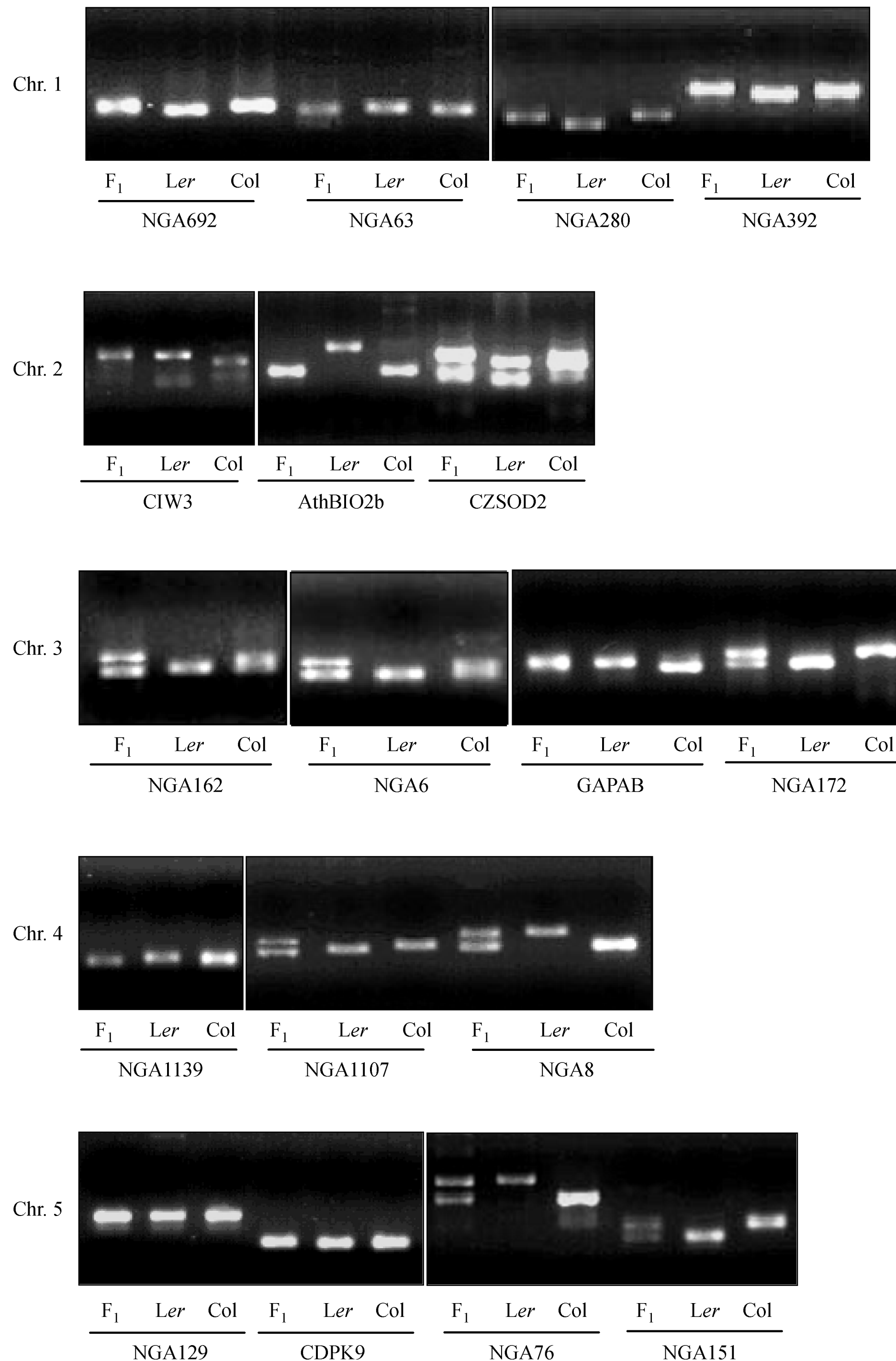

Figure 2 Results of PCR amplification using SSLP primers located on different chromosomes.

the 13:3 segregation on the basis of the χ^2 test. Among the 821 BC_1 individuals, 550 individuals displayed resistance and 271 individuals displayed susceptibility, and the ratio fit the 2:1 segregation. These results indicate that the resistance of *Arabidopsis* against *B. cinerea* infection is controlled by two genes. These two genes are suppressor genes. Only when these two genes are expressed at the same time can *Arabidopsis* display resistance to *B. cinerea*.

Selection of linked markers

Seven codominant markers located on different chromosomes of *Arabidopsis* were selected for screening of polymorphism between two bulks at first. Results showed that NGA1107 located on the fourth chromosome and NGA151 located on the fifth chromosome were polymorphic between two parents and two pools (Fig. 4), suggesting that two disease-resistant

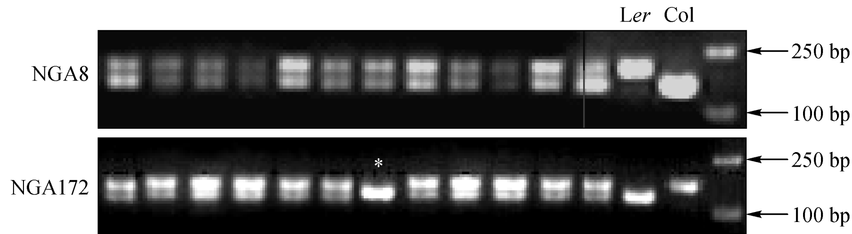

Figure 3 Results of PCR amplification with primers NGA172 and NGA8. Col means male parent; L*er* means female parent; * means non-hybrid plant.

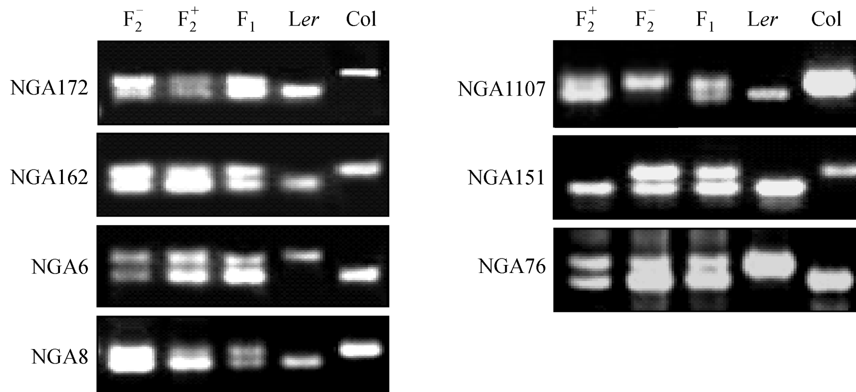

Figure 4 Choice of linkage markers. Col means male parent; L*er* means female parent; F_1 means F_1 hybrid plants; F_2^- means resistance plant pool and F_2^+ means susceptible plant pool.

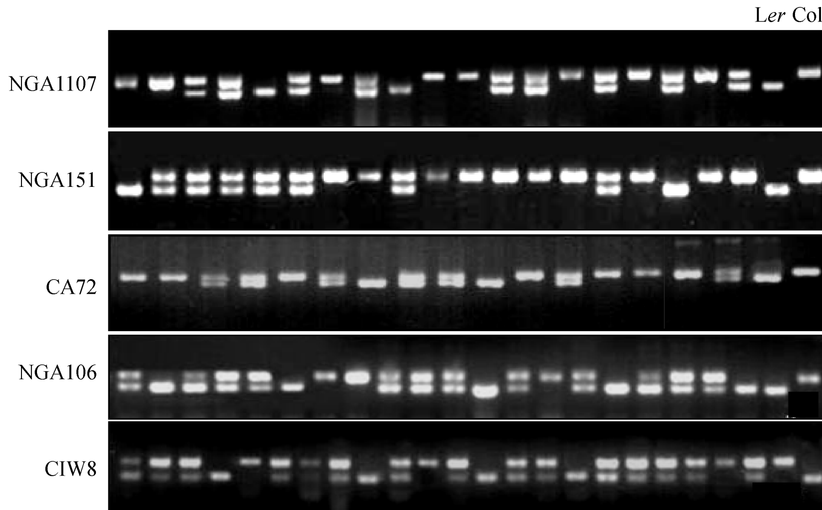

Figure 5 The segregation of partial polymorphic markers on the F_2 Populations. Col means male parent and L*er* means female parent.

genes, namely *BC1* and *BC2*, might be located on the fourth and fifth chromosome, respectively.

Chromosomal location of the resistance genes

According to the published CAPS and SSLP markers available on the internet (http://www.arabidopsis.org/home.html), markers CCR1, DHS1, R89998, NGA249, CA72, NGA106, and CIW8 closely linked to NGA1107 or NGA151 were selected to map two disease-resistant genes in 144 F_2 individuals. The analysis of CAPS and SSLP markers was performed as described previously (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). The electrophoresis bands of some F_2 plants with partial molecular markers are shown in Fig. 5.

The linkage relationship between the markers and resistant genes was analyzed using the Mapmaker/Exp Version 3.0 software. The order of the three markers and the *BC1* locus was NGA1107-CCR1-*BC1*-DHS1 with genetic distances of 1.0 cM, 1.2 cM, and 1.6 cM, respectively, for the three

intervals. The order of the five markers and the *BC2* locus was NGA249-NGA151-*BC2*-NGA106-R89998-CIW8 with genetic distances of 5.9 cM, 1.4 cM, 2.4 cM, 5.3 cM, and 5.1 cM for the five intervals, respectively. The genetic map of two resistance genes was constructed (Fig. 6).

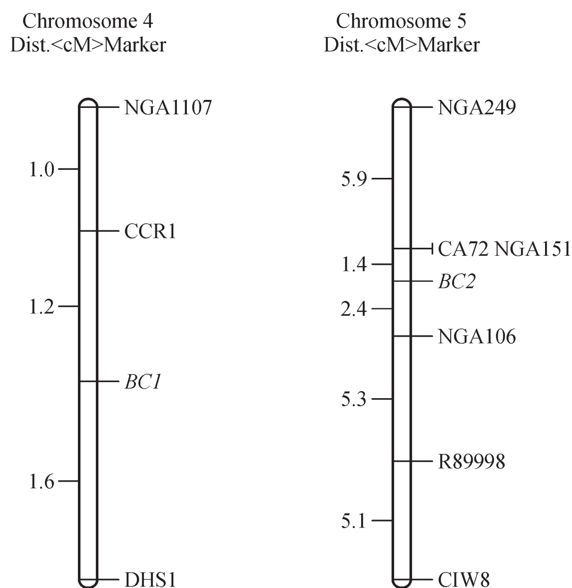

Figure 6 Linkage map of the *BC* locus on *Arabidopsis* chromosomes.

Discussion

Map-based cloning, also called positional cloning, is a method to isolate genes of interest based on an intensive genetic and physical mapping. *Arabidopsis* ecotypes Wassilewskija (Ws) and Niederzenz have both successfully been used in positional cloning projects. The most commonly used combination for mapping purposes, however, is Landsberg erecta × Columbia (L*er* × Col). Many valuable resources that can facilitate mapping L*er* × Col populations have been accumulated. According to early reports, more than 10 disease-resistant genes of *Arabidopsis* have been cloned using the positional cloning approach, including *NPR1* (Cao et al., 1997), *RPS2* (Bent et al., 1994), *RPM1* (Bisgrove et al., 1994), *RPP5* (Parker et al., 1997), *RPS4* (Gassmann et al., 1999), and *RAC1* (Borhan et al., 2004).

Map-based cloning technology also has its own limitations. When analyzing spontaneous mutations, the most frequent complex situation is a certain given character determined by more than one gene locus. For example, resistance to powdery mildew in a cross between Kashmir-1 (resistant) and Columbia (susceptible) was found to involve at least three genetic loci acting in an additive fashion (Wilson et al., 2001). In our experiment, resistance to *B. cinerea* in a cross between Col-0 (resistant) and L*er* (susceptible) types was found to involve at least two genetic loci acting in an inhibitive fashion. In these cases, fine mapping of these resistant genes requires the reduction of the genetic complexity of the mapping population, for example, by creating recombinant-inbred lines in which only one of the loci remains polymorphic.

Acknowledgements

This research was financially supported by the Natural Science Foundation of Hebei Province, China (No. C2007000539) and the “9816” Technology Projects of Agricultural University of Hebei, China.

References

AbuQamar S, Chen X, Dhawan R, Bluhm B, Salmeron J, Lam S, Dietrich R A, Mengiste T (2006). Expression profiling and mutant analysis reveals complex regulatory networks involved in *Arabidopsis* response to *Botrytis* infection. Plant J, 48(1): 28–44

Bell C J, Ecker J R (1994). Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genomics, 19(1): 137–144

Bent A F, Kunkel B N, Dahlbeck D, Brown K L, Schmidt R, Giraudat J, Leung J, Staskawicz B J (1994). *RPS*2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. Science, 265(5180): 1856–1860

Bisgrove S R, Simonich M T, Smith N M, Sattler A, Innes R W (1994). A disease resistance gene in *Arabidopsis* with specificity for two different pathogen avirulence genes. Plant Cell, 6(7): 927–933

Borhan M H, Holub E B, Beynon J L, Rozwadowski K, Rimmer S R (2004). The *Arabidopsis* TIR-NB-LRR gene *RAC*1 confers resistance to *Albugo candida* (white rust) and is dependent on *EDS1* but not *PAD4*. Mol Plant Microbe Interact, 17(7): 711–719

Cao H, Glazebrook J, Clarke J D, Volko S, Dong X (1997). The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell, 88(1): 57–63

Dhawan R, Luo H, Foerster A M, Abuqamar S, Du H N, Briggs S D, Scheid O M, Mengiste T (2009). HISTONE MONOUBIQUITINATION1 interacts with a subunit of the mediator complex and regulates defense against necrotrophic fungal pathogens in *Arabidopsis*. Plant Cell, 21(3): 1000–1019

Ferrari S, Plotnikova J M, De Lorenzo G, Ausubel F M (2003). *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2*, but not *SID2*, *EDS5* or *PAD4*. Plant J, 35(2): 193–205

Gassmann W, Hinsch M E, Staskawicz B J (1999). *The Arabidopsis RPS4* bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. Plant J, 20(3): 265–277

Konieczny A, Ausubel F M (1993). A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J, 4(2): 403–410

Lander E S, Green P, Abrahamson J, Barlow A, Daly M J, Lincoln S E, Newberg L A (1987). MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics, 1(2): 174–181

Lukowitz W, Gillmor C S, Scheible W R (2000). Positional cloning in *Arabidopsis*. Why it feels good to have a genome initiative working for you. Plant Physiol, 123(3): 795–805

Mengiste T, Chen X, Salmeron J, Dietrich R (2003). The *BOTRYTIS SUSCEPTIBLE1* gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. Plant Cell, 15(11): 2551–2565

Michelmore R W, Paran I, Kesseli R V (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA, 88(21): 9828–9832

Parker J E, Coleman M J, Szabò V, Frost L N, Schmidt R, van der Biezen E A, Moores T, Dean C, Daniels M J, Jones J D (1997). The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the toll and interleukin-1 receptors with *N* and *L6*. Plant Cell, 9(6): 879–894

Stewart C N Jr, Via L E (1993). A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques, 14(5): 748–750

The *Arabidopsis* Genome Initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature, 408 (6814): 796–815

Veronese P, Chen X, Bluhm B, Salmeron J, Dietrich R, Mengiste T (2004). The BOS loci of *Arabidopsis* are required for resistance to *Botrytis cinerea* infection. Plant J, 40(4): 558–574

Veronese P, Nakagami H, Bluhm B, Abuqamar S, Chen X, Salmeron J, Dietrich R A, Hirt H, Mengiste T (2006). The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. Plant Cell, 18 (1): 257–273

Wilson I W, Schiff C L, Hughes D E, Somerville S C (2001). Quantitative trait loci analysis of powdery mildew disease resistance in the *Arabidopsis thaliana* accession kashmir-1. Genetics, 158(3): 1301–1309

Xing J H, Ban H W, Weng Q Y, Dong J G (2010). Resistance of different *Arabidopsis* ecotypes to *Botrytis cinerea*. Journal of Agricultural University of Hebei, 6: 26–30 (in Chinese)