Marker-assisted backcrossing to introgress resistance to *Fusarium* wilt (FW) race 1 and *Ascochyta* blight (AB) in C 214, an elite cultivar of chickpea

R. K. Varshney\textsuperscript{1,2*}, S. M. Mohan\textsuperscript{1}, P. M. Gaur\textsuperscript{1}, S. K. Chamarthi\textsuperscript{1}, V. K. Singh\textsuperscript{1}, S. Srinivasan\textsuperscript{1}, N. Swapna\textsuperscript{1}, M. Sharma\textsuperscript{1}, S. Singh\textsuperscript{3}, L. Kaur\textsuperscript{3}, S. Pande\textsuperscript{1}

\textsuperscript{1}International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, 502324, India

\textsuperscript{2}CGIAR Generation Challenge Programme (GCP), c/o CIMMYT, DF, 06600, Mexico

\textsuperscript{3}Department of Plant Breeding and Genetics, Punjab Agricultural University (PAU), Ludhiana, 141004, India

*Corresponding author: r.k.varshney@cgiar.org

Telephone: 91-40-30713305

Fax: 91-40-30713074
Abstract

Fusarium wilt (FW), caused by *Fusarium oxysporum* f. sp. *ciceris* and Ascochyta blight (AB), caused by *Ascochyta rabiei* are two major constraints to chickpea production. Therefore, two parallel marker-assisted backcrossing (MABC) programmes by targeting *foc* 1 locus and two QTL regions, ABQTL-I and ABQTL-II were undertaken to introgress resistance to FW and AB, respectively in C 214, an elite cultivar of chickpea. In the case of FW, foreground selection was conducted with 6 markers (TR19, TA194, TAA60, GA16, TA110 and TS82) linked to *foc* 1 in the cross C 214 × WR 315 (FW-resistant). On the other hand, 8 markers (TA194, TR58, TS82, GA16, SCY17, TA130, TA2 and GAA47) linked with ABQTL-I and ABQTL-II were used in the case of AB by deploying C 214 × ILC 3279 (AB-resistant) cross. Background selection in both crosses was employed with evenly distributed 40 (C 214 × WR 315) to 43 (C 214 × ILC 3279) SSR markers in the chickpea genome to select plant(s) with higher recurrent parent genome (RPG) recovery. By using three backcrosses and three rounds of selfing, 22 BC$_3$F$_4$ lines were generated for C 214 × WR 315 cross and 14 MABC lines for C 214 × ILC 3279 cross. Phenotyping of these lines has identified 3 resistant lines (with 92.7-95.2% RPG) to race 1 of FW and 7 resistant lines (with 81.7-85.40% RPG) to AB that may be tested for yield and other agronomic traits under multi-location trials for possible release and cultivation.

**Keywords:** disease resistance; marker-assisted selection; molecular markers; marker-assisted backcrossing; molecular breeding.
Introduction

Chickpea (*Cicer arietinum* L.) is an important cool season food legume grown extensively by the poor farmers throughout the Indian sub-continent. India alone contributes about 67% to the global chickpea production; however, there has been a little improvement in the crop productivity (0.8 t ha\(^{-1}\)) (Gaur et al., 2012). Several biotic and abiotic stresses impose adverse effect on plants at most of the growth stages leading to low productivity. Among the biotic stresses, *Fusarium* wilt (FW) caused by soil borne fungus *Fusarium oxysporum* f. sp. *ciceris* (*foc*) and *Ascochyta* blight (AB) caused by *Ascochyta rabiei* in chickpea are the two most severe yield reducers in India and can cause complete yield losses under favourable conditions (Navas-Cortes et al., 2000; Dubey et al., 2007; Udupa and Baum 2003). AB and FW are prevalent diseases across all chickpea growing regions of the world including India. However, AB mainly occurs in north western plains due to favourable climatic conditions, while FW is mostly restricted to central and southern parts of India (Ghosh et al., 2013).

Till date, two pathotypes (yellowing and wilting) and eight pathogenic races (races 0, 1A, 1B/C, 2, 3, 4, 5, and 6) have been described for FW (Jiménez-Fernández et al., 2013; Jiménez-Gasco and Jiménez-Díaz 2003). Furthermore, genetics of five races (1A, 2, 3, 4 and 5) has also been studied (Sharma et al., 2005). In context of India, the race 1 (synonymous 1A, Indian isolate) is highly virulent in Andhra Pradesh, a major chickpea growing state of India.

To address such problems, molecular breeding strategies have been deployed in several crop species (Kulwal et al. 2011). However, availability of markers associated with trait of interest for instance, resistance to a disease is a pre-requisite for molecular breeding. In the case of FW in chickpea, molecular markers associated with resistance to six different races (0, 1A, 2, 3, 4 and 5) have been identified (see Varshney et al., 2013). These mapping studies have located resistance genes for FW races 1 (*foc 1*), 3 (*foc 3*), 4 (*foc 4*) and 5 (*foc 5*), forming a cluster, on CaLG02 (Mayer et al., 1997; Ratnaparkhe et al., 1998; Tullu et al., 1998; Winter et al., 2000; Sharma et al., 2004).

For AB resistance, high level of variability exists for the pathogenicity trait in *A. rabiei* populations and a number of pathotypes were reported (Nene and Reddy 1987). On the basis of aggressiveness of the pathogen, pathotypes have been classified mainly into two broad categories: pathotype I (less aggressive) and pathotype II (aggressive) (Chen et al. 2004).
In terms of molecular mapping, a considerable number of QTLs have been identified on several linkage groups (2, 3, 4, 6, and 8) for AB resistance in many studies (see Varshney et al., 2013). However, majority of AB resistance QTLs were reported mainly on two linkage groups namely CaLG02 and CaLG04. For instance, AB resistance QTLs namely ar1, ar2a identified by Udupa and Baum (2003) and QTLAR3 identified by Iruela et al. (2007) are present in the same genomic region mainly flanked by GA16 and TA110 markers on CaLG02 (Supplemental Figure S1). QTLs present in this genomic region confer resistance to both pathotype I and II of *A. rabiei* and contribute up to 20% phenotypic variation. This region has been referred as ABQTL-I, hereafter in the present study. Similarly, a region, referred as ABQTL-II hereafter in this study, contains QTLs, namely ar2b mapped by Udupa and Baum (2003), QTLAR1 and QTLAR2 mapped by Iruela et al. (2006) (Supplemental Figure S2). This genomic region contributes up to 34% of the phenotypic variation.

Deployment of host plant resistance is the preferred strategy for managing the above two diseases as it is economical and eco-friendly approach. Marker-assisted backcrossing (MABC) aims at conversion of targeted lines with respect to one or two traits without disturbing remaining all other native traits of target variety (Varshney et al., 2009). MABC has been successfully employed recently to introgress AB resistance with double podding traits in chickpea varieties CDC Xena, CDC Leader and FLIP98-135C (Taran et al., 2013) and a QTL-hotspot containing QTLs for root traits and abiotic stress tolerance in JG 11, a leading chickpea variety from India (Varshney et al., 2013).

Keeping in view of above, the present study employs two parallel MABC programmes that include introgression of *foc1*, resistant locus for race 1 of FW and two QTL clusters for AB resistance namely ABQTL-I (ar1, ar2a and QTLAR3) and ABQTL-II (ar2b, QTLAR1 and QTLAR2) in the genetic background of C 214, a high yielding chickpea variety but susceptible to both of the devastating diseases. Phenotypic evaluation of these MABC lines identified several lines with high level of resistance to FW and AB.
Materials and Methods

Parent Materials

C 214, a well-adapted FW and AB susceptible desi variety suitable for rainfed conditions (Bhardwaj et al., 2010; Kaur et al., 2012) was chosen as recurrent parent for introgression of resistance to FW and AB. WR 315, a desi landrace from central India resistant to race 1A and race 3 of FW (Mayer et al., 1997; Sharma et al., 2005) was selected as donor parent for introgression of genomic segment carrying \textit{foc 1} and \textit{foc 3}. For AB resistance, ILC 3279 a kabuli landrace (Udupa et al., 1998) originated from former USSR was used as donor for transferring two QTL clusters namely ABQTL-I and ABQTL-II in the present MABC programme.

Deoxynucleotid Acid Extraction

Deoxynucleotid Acid (DNA) was isolated from fresh leaves of 15-day old seedlings of the parental genotypes, \(F_1\)s and backcross progenies using the modified cetyl trimethyl ammonium bromide (CTAB) extraction method, as described in Cuc et al. (2008). Quality and quantity of DNA were checked on 0.8% agarose gel and concentration was normalized to \(~5\)ng/ul.

Polymerase Chain Reaction (PCR) and Marker Genotyping

Polymerase chain reaction for simple sequence repeat (SSR) markers from target genomic region (Table 1) and SSRs from complete genome (Varshney et al. 2013) for background selection was performed in 5 µl reaction volumes as mentioned in our earlier studies (Nayak et al., 2010; Thudi et al., 2011). Amplified PCR products were separated by capillary electrophoresis using an ABI Prism 3730 DNA Sequencer and analysed using GeneMapper® software of Applied Biosystems, USA.

Backcross Breeding

Two parallel crossing programmes namely C 214 × WR315 and C 214 × ILC 3279 were employed for generation of \(F_1\) seeds and the lines derived thereof designated as ICCX-100175 and ICCX-100176, respectively. Molecular markers associated with resistance to FW and AB were employed for identification of true hybrid plants in each cross and these plants were selected for generation of backcross progenies. Further, foreground selection for genomic regions of interest and background selection using SSR markers were employed for identification of plants.
for further backcrossing. After undertaking three rounds of backcrossing, selected plants were selfed three times (BC$_3$F$_4$) for making plants homozygous as well as multiplication of improved seeds of C 214 for FW and AB, separately.

**Phenotyping for Fusarium wilt and Ascochyta Blight**

The selected BC$_3$F$_4$ families along with their parents were sown in controlled conditions and specific to race 1 isolates at Patancheru, India during off-season (July-October 2012) in two replications using randomized complete block design (RCBD) as described by Pande et al. (2012). Data on FW reaction of the entries were recorded at 60 days after inoculation (DAI) and classified as resistant (0-20%), moderately susceptible (21-50%) and susceptible (>50%) as described by Sharma et al. (2005).

Phenotyping for AB resistance for selected BC$_3$F$_4$ families along with parental lines were screened for adult plant resistance (APR). The entries were planted in RCBD with two replications at PAU, Ludhiana, during crop season 2012-13. The experimental units were one row plots of 2 m length with 10 cm spacing between plants and 40 cm between rows. Another highly susceptible check ICC 4991 was planted after every four-test rows to provide a constant disease pressure for the AB. The disease reaction was recorded when the susceptible check showed the maximum disease severity of ‘9’ on a scale of 1-9. The lines were classified as immune (1), resistant (1.1-3.0), moderately resistant (3.1-5.0), susceptible (5.1-7.0) and highly susceptible (>7.0) as described by Kottapalli et al. (2009).
Results

Selection of Molecular Markers

For introgressing foc1 locus conferring resistance to race 1 of FW, three SSR markers (TR19, TA194, TAA60) present in the genomic region on linkage group CaLG02 and a few adjoining markers (GA16, TS82 and TA110) in the same region (Sharma et al., 2004; Sharma and Muehlbauer, 2005; Millan et al., 2006; Gowda et al., 2009) were targeted for deployment. However, after screening a total of 10 reported markers (9 SSRs and 1 allele specific associated primer) between C 214 and WR 315, 6 were polymorphic between parents and were deployed for selection of target genomic region in segregating generations.

For introgressing resistance to AB, two QTL regions namely ABQTL-I and ABQTL-II, conferring resistance for pathotype I and pathotype II of AB present on CaLG02 and CaLG04, respectively were targeted. Genomic region ABQTL-I consisting of QTL\_AR3 (Iruela et al., 2007), ar1 and ar2a (Udupa and Baum 2003), on CaLG02 contributes up to 20% phenotypic variation. Another genomic region ABQTL-II consisting of QTL\_AR1, QTL\_AR2 (Iruela et al., 2006) and ar2b (Udupa and Baum 2003) located on CaLG04 contributing up to 34% phenotypic variation was also chosen for deployment. For ABQTL-I region, out of 13 reported markers, 7 were found polymorphic between parents and only 4 markers were employed on the basis of differences in fragment sizes (bp) (TA194, TR58, TS82 and GA16) in the backcross generations. In the case of ABQTL-II region, out of 9, 5 markers were found polymorphic and 4 markers (SCY17, TA130, TA2 and GAA47) were used in the targeted cross C 214 × ILC 3279. It is important to note that one molecular marker SCY17 (Iruela et al., 2006) from the ABQTL-II region is a diagnostic marker and this marker has been given higher importance to select the plants. Details of these markers and sequences are given in Table 1.

Marker-Assisted Backcrossing for Fusarium wilt

Marker-assisted backcrossing scheme used to introgress FW resistance from WR 315 into C 214 is given in Figure 1a. C 214 (recurrent parent) was used as female and crossed with WR 315 (donor parent) as male to generate 20 F1 seeds. Out of 20 F1 plants generated and grown in the off-season in April 2010, 7 true hybrids were identified with the polymorphic markers (TAA60, TA194 and TS82) and this cross was named as ICCX-100175. All F1s were used to make first backcross (C 214 × (C 214 × WR 315) of which, 41 BC1\_F1 seeds were harvested in June 2010. All 41 BC1\_F1 seeds were sown in the off-season (starting July 2010). Further, DNA was isolated from 41 plants and
foreground selection (FGS) was done with four SSR markers namely GA16, TAA60, TA110 and TR19. Based on FGS results, 4 plants having common heterozygotes for all markers were used for second cycle of backcrossing to obtain BC$_2$F$_1$ seeds. Although molecular markers were selected for undertaking background selection (as mentioned in Table 2), because of less number of plants identified in FGS, background selection was not done with the BC$_1$F$_1$ plants.

Subsequently, second cycle of backcrossing was undertaken and 122 BC$_2$F$_1$ seeds were harvested in October, 2010. After growing 122 BC$_2$F$_1$ plants in November 2010, marker analysis with 6 markers namely GA16, TAA60, TA194, TS82, TA110 and TR19, a total of 30 BC$_2$F$_1$ plants heterozygous for all the FGS markers were selected. All 30 BC$_2$F$_1$ plants were subjected to background selection with 32 SSR markers. As a result, 5 BC$_2$F$_1$ plants having 89-95% genome recovery were selected and used for third round of backcrossing. From this backcrossing, 91 BC$_3$F$_1$ seeds were harvested in February 2011. Subsequently, after growing 91 BC$_3$F$_1$ plants in the March 2011, marker analysis with 6 markers namely GA16, TAA60, TA194, TS82, TA110 and TR19, a total of 30 BC$_3$F$_1$ plants heterozygous for all the FGS markers were selected. All 30 BC$_3$F$_1$ plants were subjected to background selection with 40 SSR markers and based on this analysis 6 BC$_3$F$_1$ plants showing 90-98% genome recovery were selfed. As a result, 86 BC$_3$F$_2$ seeds were harvested in June 2011.

A total of 18 BC$_3$F$_2$ plants with 98% of the recurrent parent genome along with the target regions were selfed to obtain a set of more than 150 BC$_3$F$_3$ seeds. A total of 146 plants obtained from this seed set were analyzed and finally, a total of 22 BC$_3$F$_4$ homozygous plants with >98% genetic background recovery were selected based on marker analysis. Details about number of plants analyzed in foreground and background selection, number of plants found heterozygous/ homozygous and used for next generation for crossing or for generation advancement have been provided in Table 2.

**Marker-Assisted Backcrossing for Ascochyta Blight**

In the MABC programme for AB (Figure 1b), C 214 (recurrent parent), was used as female and crossed with ILC 3279 (donor parent) as male to generate 20 F$_1$ seeds. Out of 20 F$_1$ plants generated and grown in the off-season (starting in April 2010), 6 true hybrids were confirmed with the markers (GAA47 and TA130). All 6 true F$_1$ plants were used to make the first backcross (C 214 × (C 214 × ILC 3279) and 38 BC$_1$F$_1$ seeds were harvested in June,
2010. All 38 BC₁F₁ seeds were sown in the off-season (starting July 2010). Genotyping of 38 BC₁F₁ plants with one SSR marker (GA16) for ABQTL-I region of CaLG02 and 3 SSR markers (TA130, TA2 and GAA47) for ABQTL-II region located on CaLG04 identified only 2 BC₁F₁ plants showing heterozygosity for all the markers for both ABQTL-I and ABQTL-II regions. These two plants were selected for second cycle of backcrossing. Subsequently, second cycle of backcrossing was undertaken using pollen from above selected 2 BC₁F₁ plants and 124 BC₂F₁ seeds were harvested in October, 2010. After growing 124 BC₂F₁ plants in the main crop season (starting in November 2010), initially all 124 BC₂F₁ plants were screened with the diagnostic marker SCY17 and 60 BC₂F₁ plants were selected as positive for the this marker. These plants were further screened with 6 markers namely TA194, TS82 and GA16 from ABQTL-I region and TA130, TA2 and GAA47 from ABQTL-II region. As a result, 46 BC₂F₁ plants heterozygous for all markers i.e. both QTL regions were selected. All 46 BC₂F₁ plants were subjected to background selection with 29 SSR markers. On the basis of background selection, six BC₂F₁ plants with 80-87% recurrent parent genome (RPG) recovery were selected and used for third round of backcrossing. As a result, a total of 88 BC₃F₁ seeds were harvested in February 2011. Subsequently, after growing 88 BC₃F₁ seeds in the off-season (starting March 2011), marker analysis was initially done with SCY17 marker and 38 BC₃F₁ plants were found positive for SCY17 marker. Subsequently, these 38 BC₃F₁ plants were screened with 6 markers namely TA194, TS82, GA16 from ABQTL-I region and TA130, TA2 and GAA47 from ABQTL-II regions for background selection with 43 SSR markers. Although 23 BC₃F₁ plants showed 80-90% genome recovery in background selection, all 38 BC₃F₁ plants positive for the diagnostic marker (SCY17) were used for selfing to obtain more number of seeds. Therefore, a total of 166 BC₃F₂ seeds were harvested in the month of June 2011. After growing 51 BC₃F₂ plants, in the first instance, in the month of July 2011, and a total of 9 BC₃F₂ plants were selected on the basis of phenotypic similarity to the recurrent parent to generate BC₃F₃ generation. These plants have been selfed further to generate more than 200 BC₃F₃ seeds. A total of 189 BC₃F₃ plants from this generation were analyzed and finally 48 homozygous lines showing more than 85% genome recovery were selected to obtain BC₃F₄ seeds. Although 48 BC₃F₃ lines were selected, seeds from only fourteen lines could be harvested (from green house in 2012) due to poor seed set. On the basis of phenotypic data (see later), seven resistant plants were identified and analyzed with foreground markers of both QTL regions and background data with SSR markers reflected RPG recovery ranged from (81.7 to 85.4%).
Further on the basis of foreground selection, it was revealed that five plants were found positive for ABQTL-I region and three plants were found positive for ABQTL-II region. However, only one plant (ICCX-100176-470-2-16) was found positive for both QTL regions. Details of each activity viz. number of seeds generated, plants analyzed for foreground and background selection, number of plants found heterozygous/ homozygous and used for next generation have been given in Table 3.

**Phenotyping of Marker-Assisted Backcrossing Lines for Resistance to Fusarium wilt**

All selected 22 BC$_3$F$_4$ progenies were grown and subjected to phenotyping against race 1 of FW under controlled conditions at ICRISAT in the off-season (July-October 2012). Of these, three progenies namely ICCX-100175-349-2-2, ICCX-100175-382-4-6 and ICCX-100175-389-3-2 showed high to moderate level of resistance (Figure 2 and Table 4). One of these three progenies namely ICCX-100175-349-2-2 has not shown any wilt symptoms i.e., complete resistance (100%) while the other two progenies have shown 95% (ICCX-100175-389-3-2) and 80% (ICCX-100175-382-4-6) resistance at sixty days after inoculation.

**Phenotyping of Marker-Assisted Backcrossing Lines For Resistance to Ascochyta Blight**

Fourteen BC$_3$F$_4$ lines homozygous for ABQTL-I and ABQTL-II regions were used for AB screening under field conditions (Kaur et al. 2011) during the main crop season 2012-13 at PAU, Ludhiana (disease hotspot region of India). Of fourteen lines tested in the field, a total of seven BC$_3$F$_4$ lines (Table 5) showed resistance reaction (Figure 3). Out of 7 lines, 4 lines showed resistant score of 2; and 3 lines showed resistant score of 3, in comparison to a score of 7 and 4 of recurrent (C 214) and donor parent (ILC 3279), respectively. The lines possess either of the genomic regions showed higher level of resistance compared to ICCX-100176-470-2-16 line possessing both QTL regions.

**Molecular Analysis of Carrier Chromosomes in Marker-Assisted Backcrossing Lines**

To analyze the recovery of recurrent parent genome on the carrier chromosomes namely CaLG02 and CaLG04, SSR markers present on these chromosomes were used to analyze backcross progenies for the respective chromosomes. In the case of FW resistant progenies, 14 additional SSR makers (other than those that were used for FGS and BGS) were used for parental polymorphism survey between C 214 and WR 315. Out of 14, only 2 SSR markers were found polymorphic between parents, and used for identification of donor parent genome. Due to identification of
less number of polymorphic markers, all three improved lines showed similar pattern with the nearest flanking marker TA103 (Figure 4A). Interestingly, the next marker, H1F05 showed donor parent alleles in all three improved lines.

In the case of AB resistance progenies, additional 24 and 32 SSR markers corresponding to CaLG02 (for ABQTL-I) and CaLG04 (for ABQTL-II) regions were targeted for analyzing donor parent genome introgression on carrier chromosome in BC$_3$F$_4$ lines. Screening of 24 and 32 markers on parental lines showed 7 and 6 markers polymorphic in CaLG02 and CaLG04, respectively. Genotyping of BC$_3$F$_4$ lines with these polymorphic markers identified superior recombinant lines for ABQTL-I and ABQTL-II regions. On the basis of recurrent parent genome recovery on carrier chromosomes, one improved line ICCX-100176-470-2-5, with lesser introgression of donor parent genome in both the chromosomes (CaLG02 and CaLG04) (Figure 4B and 4C) was identified that may be used for further detailed evaluation in multi-location trials.
Discussion

FW and AB are two most devastating diseases of chickpea causing severe yield losses. Conventional method of breeding for disease resistance is tedious and time taking process. However, MABC applying foreground selection using QTL linked markers and background selection using genome-wide SSR markers for recovery of recurrent parent genome (RPG) is an environment independent, precise and quick approach for the development of cultivars for the trait of interest (Varshney et al. 2010). This study reports successful introgression of resistance to FW and AB in the genetic background of C 214.

QTL mapping identified resistance loci with flanking molecular markers for resistance to a number of races of FW viz. foc 0 locus for race 0 (Cobos et al. 2005), foc 1 locus for race 1 (Gowda et al., 2009), foc 2 locus for race 2 (Gowda et al., 2009), foc 3 locus for race 3 (Sharma et al., 2004; Gowda et al. 2009), foc 4 locus for race 4 (Winter et al., 2000; Sharma et al., 2004; Sharma and Muelbauer, 2005) and foc 5 locus for race 5 (Cobos et al., 2009). Recently two novel QTLs (FW-Q-APR-6-1 and FW-Q-APR-6-2) for FW for race 1 explaining 10.4–18.8% of phenotypic variation have also been reported (Sabbavarapu et al., 2013). Similarly, a large number of QTLs for AB resistance were reported namely ar2b (Udupa and Baum, 2003), QTL_AR3 (Iruela et al., 2007), ar1 (Iruela et al., 2007), ar2 (Iruela et al., 2007) etc. Recently, we have mapped six QTLs for AB resistance explaining up to 31.9% of phenotypic variation (Sabbavarapu et al., 2013).

In view of above, foc 1 locus conferring resistance to race 1 of FW, and two QTL regions (ABQTL-I and ABQTL-II) for AB resistance located on two different LGs were targeted for introgression into the recurrent parent C 214. Foreground selection with QTL linked markers and background selection using genome-wide SSR markers were employed in each backcross generation to select positive plants for crossing or selfing. However, in case of FW, due to identification of only four BC1F1 plants positive for all foreground markers, background selection was not imposed. In the BC2F1 generation, five plants with recurrent parent genome recovery ranging from 89-95% were identified based on SSR analysis in comparison to 87.5% of expected average similarity. Following foreground and background selection with molecular markers, several plants up to 98% of similarity were developed as early as in BC3F1 generation. Similarly, in the case of AB in BC1F1 generation, due to identification of only two plants positive for all markers from both QTL regions, ABQTL-I and ABQTL-II, background selection was not imposed. However, the BC2F1 generation had plants up to 87% of RPG as expected (87.5%). However, plants with 80-90%
RPG were identified in BC$_3$F$_1$ generation, further based on resistance reaction seven plants were selected in BC$_3$F$_3$ generation which showed the RPG recovery ranged from 81.7 to 85.4%. The lower recovery of recurrent genome of selected plants was might be fixations of heterozygous alleles at BC$_3$F$_1$ generation towards donor parent genome.

To analyze the recovery of recurrent parent alleles on carrier chromosomes in BC$_3$F$_4$ MABC lines, carrier chromosome specific (CaLG02 for FW cross, CaLG02 and CaLG04 for AB cross) polymorphic SSR markers were also used to identify recombinant lines with lesser donor parent segments in both crosses. However, due to lesser number of polymorphic markers identified between C 214 × WR 315, the real selection of lines in the case of MABC progenies for FW was not effective. Analyzing MABC for AB resistance lines using carrier chromosome specific markers for two LGs (CaLG02 and CaLG04) in C 214 × ILC 3279, one line namely ICCX-100176-470-2-5, was identified with lesser donor parent introgression in both the carrier chromosomes. This line with lesser donor parent introgression will be used for further evaluations for other important traits.

Phenotyping for FW resistance of MABC and parental lines showed resistance reaction in sick plot nursery. However, three MABC lines showed resistance reaction from 0% to 20% of FW resistance reaction in comparison to C 214 (recurrent parent) of 54.5% and 6% of WR 315 (donor parent). Of three, two lines have shown better resistance ICCX-100175-349-2-2 (0% of FW incidence) and ICCX-100175-389-3-2 (5% of FW incidence). It is also important to mention here that the introgressed segment in C 214 also carries $foc$ 3 locus, resistance to race 3 of FW. Therefore, the MABC lines generated in this study may show resistance to race 3 of FW as well. Phenotypic evaluation, in the target region of FW race 3, however needs to be undertaken to confirm above mentioned speculation.

Similarly, MABC lines for AB resistance showed disease reaction score <3.0 in comparison to 7 and 4 of recurrent (C 214) and donor parent (ILC 3279), respectively on 1-9 scale. However, one line identified with minimum donor parent chromosome namely ICCX-100176-470-2-5 on both of the carrier chromosome showed high level of resistant reaction (score of 2), in comparison to donor parent ILC 3279 (score of 4) and recurrent parent C 214 (score of 7). Surprisingly, one line which was positive for both the QTL regions (ABQTL-I and ABQTL-II) showed resistant reaction score of 3, in comparison to lines possessing either ABQTL-I or ABQTL-II with resistant score of 2, except ICCX-100176-470-3-3, which showed resistant reaction of 3 and possess only ABQTL-I.
In the present study MABC lines with single genomic region (either ABQTL-I or ABQTL-II) compared to that of a line having both ABQTL-I and ABQTL-II regions showed higher level of resistance to AB. This may be due to antagonistic epistatic interaction of two genomic regions that was also evident by the disease reaction of donor parent ILC 3214, which has comparatively low level of resistance as compared to MABC lines (Jannick, 2009). Similar observation was made by Castro et al. (2003) that during the transfer of resistant QTLs for barley strip rust into the elite background, presence of single QTL alleles in lines showed higher level of resistance in comparison to presence of two QTL alleles, and they explained it may due to double crossover between markers and disease resistance loci, undetected resistance genes and/or incomplete penetrance. Therefore, more experiments are necessary to determine the specific role of each QTL in this study. However, based on our experiments it can be concluded that either ABQTL-I or ABQTL-II can be targeted for development of AB resistant breeding lines.

In summary, this study demonstrates use of MABC to develop superior lines with enhanced resistance to race 1 (and possibly race 3) of FW and AB. These lines may be used for multi-location field trials of All India Coordinated Research Project (AICRP) on Chickpea of Indian Council of Agricultural Research (ICAR) in India for possible release of most promising MABC lines as improved variety for commercial cultivation. However, to add value further, intercrossing may be undertaken using superior MABC lines for FW and AB resistance, developed in the present study. Pyramided lines for resistance to FW (foc 1 and possibly foc 3) and AB (ABQTL-I and ABQTL-II) are expected to perform better in different agro-climatic zones. Therefore, it is planned to undertake intercrossing of FW and AB-resistant lines and then selfing of the products of intercrossing to make them homozygous. Homozygous and pyramided version of C 214 subsequently will be evaluated for yield and yield related traits in disease hot-spot locations of India. After due testing, superior lines will be tested further under AICRP on Chickpea of ICAR for release of improved variety with enhanced resistance to FW and AB for commercial cultivation.

Acknowledgments

The authors are thankful to B. Vinay Kumar, B.V. Rao, T. Rameshwar and Md. Aziz for their technical help. Authors also appreciate useful discussions held with Mahendar Thudi, Manish K. Pandey, Pawan Khera and Manish Roorkiwal in various ways. The present study was supported under the Accelerated Crop Improvement Programme (ACIP) from the Department of Biotechnology (DBT), Government of India. This work has been undertaken as part of the CGIAR Research Program on Grain Legumes. ICRISAT is a member of CGIAR Consortium.
References


**Legend to Figures**

**Figure 1. Scheme of marker assisted backcrossing (MABC) deployed.**

To introgress *foc1* locus conferring resistance to race 1 of *Fusarium* wilt (FW) and two QTL regions (ABQTL-I and AB-QTL-II) conferring resistance to *Aschocyta* blight (AB), two different donors namely WR 315 (FW- resistant) and ILC 3279 (AB resistant) were crossed separately with C 214, recurrent parent. F₁ seeds generated from these crosses were sown and after marker analysis for heterozygosity, positive plants were used for making the backcrosses. Subsequently, in backcross generation, molecular markers were used for foreground and background selection. In total, three backcrosses and three rounds of selfing were undertaken to develop homozygous introgression lines.

**Figure 2. Screening of marker-assisted backcrossing (MABC) lines for resistance to *Fusarium* wilt.**

Phenotyping of BC₃F₄ lines for resistance to the race 1 of FW under controlled conditions identified three lines that showed resistance reaction, similar to donor parent. In the same experiment, known susceptible check showed highly susceptible reaction during artificial inoculation.

**Figure 3. Screening of marker-assisted backcrossing (MABC) lines for resistance to *Ascochyta* blight.**

BC₃F₄ lines containing QTLs for AB resistance along with parental lines were screened in artificial epiphytotic field conditions at Punjab Agricultural University (PAU), Ludhiana (India). Recurrent parent C 214 was completely died in the field conditions, showed presence of highly virulent isolate in the field conditions. Three MABC lines, however, showed resistant reaction to the virulent isolate.

**Figure 4. Graphical genotypes of selected lines using SSR markers for the carrier chromosomes for MABC lines for resistance to FW and AB**

A. Polymorphic SSR markers on the carrier chromosome (CaLG02) between parental lines (C 214 × WR 315) were used to analyze the introgression of donor parent genome associated with resistance loci *foc1*. It is evident that all 3 BC₃F₄ lines showed expected graphical genotypes (GGT).

B. Graphical genotypes (GGT) were generated after genotyping MABC lines for ABQTL-I with CaLG02 specific markers that showed polymorphism between C 214 and ILC 3279.
C. MABC lines for ABQTL-II region were genotyped with CaLG04 specific markers that showed polymorphism between C 214 and ILC 3279. The genotyping data was used for preparation of GGT. In each case, the GGT identified the plants with minimum amount of the donor parent genome.

Supplemental Figure S1. Representative nomenclature for ABQTL-I on CaLG02 showing position of QTL AR3 as per Iruela et al. 2007 (Cross: ILC 3279 × WR 315) and ar1/ar2a as per Udupa and Baum (2003) (cross: ILC 1272 × ILC 3279) contributing up to 20% of phenotypic variation.

Supplemental Figure S2. Representative nomenclature for ABQTL-II on CaLG04 (QTL AR1 / QTL AR2 as per Iruela et al. (2006) (cross: ILC 3279 × WR 315) and ar2b as per Udupa and Baum (2003) (cross: ILC 1272 × ILC 3279) contributing up to 34% phenotypic variation.

Table 1 Details on molecular markers used for undertaking foreground selection in marker-assisted backcrossing (MABC) programmes for Fusarium wilt (FW) and Ascochyta blight (AB)
### Table 2

Details on genotyping, selection and crossing of lines in different generations during marker-assisted backcrossing (MABC) for introgressing resistance to race 1 (*foc1*) in C 214

<table>
<thead>
<tr>
<th>Lines</th>
<th>BC$_1$F$_1$</th>
<th>BC$_2$F$_1$</th>
<th>BC$_3$F$_1$</th>
<th>BC$_3$F$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA16</td>
<td>41</td>
<td>38</td>
<td>122</td>
<td>8</td>
</tr>
<tr>
<td>TAA60</td>
<td>41</td>
<td>41</td>
<td>23</td>
<td>122</td>
</tr>
<tr>
<td>TA194</td>
<td>41</td>
<td>-</td>
<td>-</td>
<td>122</td>
</tr>
<tr>
<td>TS82</td>
<td>41</td>
<td>-</td>
<td>-</td>
<td>122</td>
</tr>
<tr>
<td>TA110</td>
<td>41</td>
<td>27</td>
<td>13</td>
<td>122</td>
</tr>
<tr>
<td>TR19</td>
<td>41</td>
<td>40</td>
<td>24</td>
<td>122</td>
</tr>
</tbody>
</table>

Heterozygotes in the case of BC$_1$F$_1$, BC$_2$F$_1$, BC$_3$F$_1$, for undertaking background selection and homozygotes in the case of BC$_3$F$_2$ & BC$_3$F$_3$

<table>
<thead>
<tr>
<th>Markers used in foreground selection and plants selected during different generations</th>
<th>Analyzed</th>
<th>Scorable bands</th>
<th>Heterozygotes</th>
<th>Analyzed</th>
<th>Scorable bands</th>
<th>Heterozygotes</th>
<th>Analyzed</th>
<th>Scorable bands</th>
<th>Heterozygotes</th>
<th>Analyzed</th>
<th>Scorable bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA16</td>
<td>41</td>
<td>38</td>
<td>8</td>
<td>122</td>
<td>106</td>
<td>38</td>
<td>91</td>
<td>85</td>
<td>29</td>
<td>86</td>
<td>78</td>
</tr>
<tr>
<td>TAA60</td>
<td>41</td>
<td>41</td>
<td>23</td>
<td>122</td>
<td>121</td>
<td>28</td>
<td>91</td>
<td>86</td>
<td>29</td>
<td>86</td>
<td>85</td>
</tr>
<tr>
<td>TA194</td>
<td>41</td>
<td>-</td>
<td>-</td>
<td>122</td>
<td>121</td>
<td>54</td>
<td>91</td>
<td>81</td>
<td>18</td>
<td>86</td>
<td>85</td>
</tr>
<tr>
<td>TS82</td>
<td>41</td>
<td>-</td>
<td>-</td>
<td>122</td>
<td>118</td>
<td>55</td>
<td>91</td>
<td>84</td>
<td>29</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td>TA110</td>
<td>41</td>
<td>27</td>
<td>13</td>
<td>122</td>
<td>86</td>
<td>39</td>
<td>91</td>
<td>84</td>
<td>25</td>
<td>86</td>
<td>78</td>
</tr>
<tr>
<td>TR19</td>
<td>41</td>
<td>40</td>
<td>24</td>
<td>122</td>
<td>89</td>
<td>42</td>
<td>91</td>
<td>88</td>
<td>27</td>
<td>86</td>
<td>81</td>
</tr>
</tbody>
</table>

| Number of SSR markers used for background selection | * | 32 | 40 |
| Number of plants after background selection (with % recurrent parent genome recovery) | * | 15 | (80-95%) | 6 | (90-98%) |
| Number of plants selected with higher background genome recovery used for generation advancement | 4 | 5 | 6 |

* = Background selection was not done due to less number of plants; † = Plants were selected based on priority, - = not done

### Table 3

Disease reaction of parental and BC$_3$F$_4$ lines carrying *foc1* locus conferring resistance to race 1 of *Fusarium oxysporum*

<table>
<thead>
<tr>
<th>Lines</th>
<th>FW incidence (%)</th>
<th>Disease reaction$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C 214 (recurrent parent)</td>
<td>54.50</td>
<td>Susceptible</td>
</tr>
<tr>
<td>WR 315 (donor parent)</td>
<td>6</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

$^\dagger$ = Plants were selected based on priority, - = not done
MABC lines
ICCX-100175-349-2-2 0 Resistant
ICCX-100175-389-3-2 5 Resistant
ICCX-100175-382-4-6 20 Resistant

\*FW disease reaction of each line was scored as per the scale of Sharma et al. (2005). The plants were categories as resistant (0-20%), moderately susceptible (21-50%) and susceptible (>50%)

Table 4 Details on genotyping, selection and crossing of lines in different generations during marker-assisted backcrossing (MABC) for introgressing Ascochyta blight (AB) resistance in C 214

<table>
<thead>
<tr>
<th>Targeted QTL region</th>
<th>Markers</th>
<th>BC1F1</th>
<th>BC2F1</th>
<th>BC3F1</th>
<th>BC4F1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Analyzed</td>
<td>Scorable bands</td>
<td>Heterozygotes</td>
<td>Analyzed</td>
</tr>
<tr>
<td>ABQTL-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA194</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>TRS2</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TS82</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>GA16</td>
<td></td>
<td>38</td>
<td>37</td>
<td>14</td>
<td>60</td>
</tr>
<tr>
<td>Common heterozygotes / homozygotes for all ABQTL-I markers</td>
<td></td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>ABQTL-II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCY17</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>124</td>
</tr>
<tr>
<td>TA130</td>
<td></td>
<td>38</td>
<td>38</td>
<td>21</td>
<td>60</td>
</tr>
<tr>
<td>TA2</td>
<td></td>
<td>38</td>
<td>28</td>
<td>19</td>
<td>60</td>
</tr>
<tr>
<td>GAA47</td>
<td></td>
<td>38</td>
<td>29</td>
<td>14</td>
<td>60</td>
</tr>
<tr>
<td>Common heterozygotes / homozygotes for all ABQTL-II markers</td>
<td></td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 5 Disease reaction of parental and BC3F4 lines carrying two QTL regions (ABQTL-I and ABQTL-II) conferring resistance to Ascochyta blight

\* = Although foreground and background selection was done, all 38 plants which were positive with SCAR marker (SCY17) for selfing to obtain more number of plants; † = Plants were selected based on the priority; NA = not available
<table>
<thead>
<tr>
<th>Lines</th>
<th>ABQTL-I</th>
<th>ABQTL-II</th>
<th>AB score</th>
<th>Disease reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C 214 (recurrent parent)</td>
<td>--</td>
<td>--</td>
<td>7</td>
<td>Susceptible</td>
</tr>
<tr>
<td>ILC 3279 (donor parent)</td>
<td>++</td>
<td>++</td>
<td>4</td>
<td>Moderately resistant</td>
</tr>
<tr>
<td>MABC lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICCX-100176-421-1-11</td>
<td>--</td>
<td>++</td>
<td>3</td>
<td>Resistant</td>
</tr>
<tr>
<td>ICCX-100176-421-1-12</td>
<td>--</td>
<td>++</td>
<td>2</td>
<td>Resistant</td>
</tr>
<tr>
<td>ICCX-100176-470-2-5</td>
<td>++</td>
<td>--</td>
<td>2</td>
<td>Resistant</td>
</tr>
<tr>
<td>ICCX-100176-470-2-7</td>
<td>++</td>
<td>--</td>
<td>2</td>
<td>Resistant</td>
</tr>
<tr>
<td>ICCX-100176-470-2-16</td>
<td>++</td>
<td>++</td>
<td>3</td>
<td>Resistant</td>
</tr>
<tr>
<td>ICCX-100176-470-3-1</td>
<td>++</td>
<td>--</td>
<td>2</td>
<td>Resistant</td>
</tr>
<tr>
<td>ICCX-100176-470-3-3</td>
<td>++</td>
<td>--</td>
<td>3</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

++ Indicates the presence of marker alleles from donor parent at QTL region, -- Indicates the presence of marker alleles from recurrent parent at QTL region. AB disease scoring of each lines was on a scale of 1-9 (1 = immune, 1.1–3.0 = resistant, 3.1–5.0 = moderately resistant, 5.1–7.0 = susceptible, >7 = highly susceptible (Kottapalli et al. 2009).
**Diagram (a):**

- **C 214 x WR 315**
  - **F₁**
    - Out of 20, 7 F₁ plants were true hybrids and generated 41 BC₁F₁ seeds (April 2010 to July 2010)
  - **BC₁F₁**
    - Out of 41, 4 BC₁F₁ plants identified through MAS and 122 BC₂F₁ seeds were generated (July 2010 to October 2010)
  - **BC₂F₁**
    - Five, out of 122 BC₂F₁, were identified positive, through MAS were used to generate 91 BC₃F₁ seeds (November 2010 to February 2011)
    - **BC₃F₁**
      - Of 91 plants analyzed, 6 BC₃F₁ were selected through MAS for generation of 86 BC₂F₂ seeds (March 2011 to June 2011)
      - **BC₂F₂**
        - Out of 86, 18 plants were selected through MAS to generate 146 seeds (July 2011 to October 2011)
        - **BC₂F₃**
          - Out of 146, 22 BC₂F₃ plants were selected through MAS (October 2011 to February 2012)
          - **BC₃F₄**
            - Artificial screening in greenhouse against race 1 of *Fusarium* wilt, identified three resistant lines (July-October 2012)

**Diagram (b):**

- **C 214 x ILC 3279**
  - **F₁**
    - 20 F₁ analyzed, 6 F₁ were identified true hybrids to generate 38 BC₁F₁ seeds (April 2010 to July 2010)
  - **BC₁F₁**
    - Out of 38, 2 BC₁F₁ heterozygous for all markers, selected for generation of 124 BC₂F₁ seeds (July 2010 to October 2010)
    - **BC₂F₁**
      - Analysis of 124 BC₂F₁ plants using Foreground and background markers identified 6 plants for generation of 88 BC₃F₁ seeds (November 2010 to February 2011)
      - **BC₃F₁**
        - Out of 88 plants 38 BC₃F₁ were selected to generate 51 BC₂F₂ plants through MAS (March 2011 to June 2011)
        - **BC₂F₂**
          - Out of 51 plants, 9 BC₂F₂ were selected to generate 189 BC₃F₃ through MAS (July 2011 to October 2011)
          - **BC₃F₃**
            - Out of 189 plants analyzed, 48 BC₃F₃ were selected through MAS (October 2011 to February 2012)
            - **BC₄F₄**
              - Disease screening of 48 lines against *Ascochyta* blight, identified seven resistant lines in field at PAU, Ludhiana (Crop-season 2012-13)