DEVELOPMENT OF MOLECULAR MARKER LINKED TO CLUBROOT (*PLASMODIOPHORA BRASSICAE WOR.)* RESISTANCE GENE AND ITS APPLICATION IN CHINESE CABBAGE (*BRASSICA RAPA* L.) BREEDING

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Abstract

Random amplified polymorphic DNA (RAPD) marker RA12-75A linked to a clubroot-resistance locus of Brassica rapa L. was converted into co-dominant marker SCARp91F-SCARp636R useful for differentiation of susceptible and resistant genotypes in populations from cross of lines of European turnip (ECD04-J - donor of resistance genes) and Chinese cabbage. But, ineffectiveness both RAPD locus RA12-75A, and the developed SCARp91F-SCARp636R marker was shown for hybrid population obtainedfrom resistant and susceptible plants of turnip.

Clubroot of cabbage caused by plant pathogen *Plasmodiophora brassicae* Wor. is one of the most damaging diseases of Brassica crops including *Brassica rapa* L. [3]. The pathogen affects root system and causes swelling of parts of the roots, giving rise to the characteristic "clubs" [9]. Severely distorted roots are unable to absorb and distribute mineral and nutrients and water from the soil. In mild cases wilting and stunting occurs, and in severe cases, the plant dies [1]. Cultural practices, especially the application of calcium and boron, and lime to decrease the pH of the soil, may reduce disease pressure but these measures are often not sufficient to keep the crop healthy [14]. Clubroot control by means of crop rotation does not offer much promise because the spores can remain infectious for many years and cruciferous weeds maintain the disease presence. The use of pesticides is restricted due to lack of economically effective and environmental friendly fungicide [7]. Thus, the breeding and introduction of resistant cultivars is one of the most effective approaches to minimize crop loss from infection with this pathogen.

Several breeding programs have been successfully carried out to develop clubroot resistante (CR) cultivars of Chinese cabbage using European turnips as sources of resistance [5, 15, 10]. Among turnip genotypes, clubroot resistance is under the control of three independent dominant genes [12, 4].

Infection of some CR cultivars has been reported in some production areas [10] indicating a breakdown of the introduced CR trait. Breeding of more resistant CR cultivars would require the accumulation of three CR genes in a single genotype. Simple backcross method is effective to incorporate a single CR locus to Chinese cabbage, however incorporation more than two CR loci is rather difficult. Pyramiding CR genes with MAS may be an ideal strategy for this complex breeding procedure [8, 11].

Analysis of segregating double haploid population derived from a cross between clubroot resistant turnip and clubroot susceptible Chinese cabbage enabled to obtain three random amplified polymorphic DNA (RAPD) markers linked to the major CR gene [11].

All markers were checked in Center "Bioingenering" (Russia) on segregating populations BCi and F_2 derived from a cross between CR turnip and susceptible Chinese cabbage. Effectiveness and usefulness for marker assisted selection of marker RA12-$75A_{65}O$ was confirmed. However there are problems with RAPD markers associated with poor reproducibility, stability and also typical dominance character. RAPD markers convert to PCR markers that can be detected more easily and reliably, and are designate as sequence characterized amplified regions (SCARs).

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In the present study, we focused on the development of a reliable method for converting RAPD clubroot resistance gene marker RA12-75650 into dominant\codominant SCAR markers and the evaluation of their effectiveness for Chinese cabbage and turnip marker assisted selection.

Materials and methods

Plant materials

A CR turnip inbred line ECD04-1 was used as a donor of clubroot resistance genes. Highly susceptible to clubroot turnip inbred line JRT1-1 and Chinese cabbage inbred line Ce-3 - as donors of susceptibility alleles. The F_2 and BCl[8] populations derived from crossing ECD04-lxJRTl-l and ECD04-1 x Ce-3 were used for genetic analysis.

Clubroot disease resistance tests

Field isolate detected to be race 16/11/31 of clubroot on the basis of Buczacki's classification [6] was used to evaluate the responses of F_2 and BC| families. The galls from Chinese cabbage for maintaining were collected and stored at -20°C until use. The spores concentration of inoculum was adjusted to 1 x10^7 spores/ml. Seeds from the corresponding families were planted in 64-well multipots and maintained in the greenhouse.

Three-day-old seedlings were inoculated by pipette method applying 5 ml of spore suspension to each well [13]. Symptoms of disease were scored after 6 weeks on a 0-3 scale (Buczacki et al. 1975). Plants in classes 0 and 1 were considered resistant and those in classes 2 and 3 as susceptible.

DNA isolation and amplification

Total DNA was isolated from young leaves of plants according to the method described by D.Dorokhov and E.Kloke (1997) [2].

For the detection of random amplified polymorphic DNA (RAPD), 20 ng genomic DNA, lx buffer (67 mmol Tris-HCl pH-8,8; 166 mmol $(NH_4)2S0_4$; 2,5 mmol MgCb; 0,01% Tween-20), 0.5 U Taq DNA polymerase, 0.2 mmol of each dNTP and 5-15 pmol of arbitrary primer RA12-75 - 5'- C ATT ATGCGGGC -3'.

The reaction was performed using a GeneAmp PCR system 2000 (Applied Biosystems) with the following parameters: 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 30 s, 36°C for 30 s and 72°C for 1 min; an extra extension at 72°C for 10 min. The annealing temperature for SCAR primers was 64°C.

The amplified products were analyzed by electrophoresis in 1,5 % agarose gel containing 0,5 μg\ml ethidium bromide.

Cloning of the amplified band

The amplified fragment of the expected size was cut from the gel and extracted using extraction kit. The eluted RAPD fragment was cloned into the pGEMT Vector System 1 (Promega Corporation).

Plasmid DNA isolated from more than ten clones was amplified with the corresponding selective primer combination to confirm clones containing the correct RAPD marker. Clones with inserts of the same size as that of the RAPD marker were selected.

The resultant plasmids were sequenced with an ABI 3100 Genetic Analyzer (Applied Biosystems Co.). Analysis of sequence information was performed using the BLAST search with GenBank (http://www.ncbi.nlm.nih.gov/GenBank). Primer sequences suitable for the specific amplification were designed using OL1GO 6.1 (Molecular Biology Insights, Cascade, USA) software.

Results

Survey of lines belonging to Brassica rapa

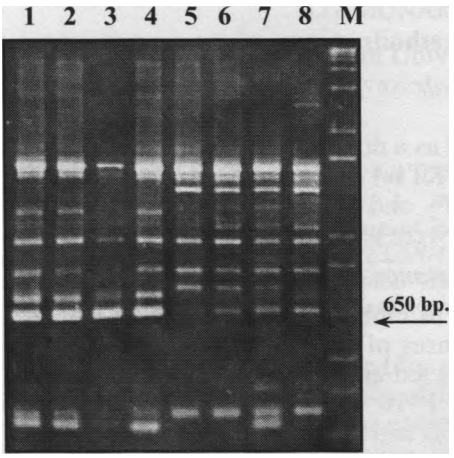

Fig. 1. DNA amplification using arbitrary primer RA12-75 (5-CATTATGCGGGC-3'). Arrow indicate the CR marker, M - molecular marker, Lanes: 1-4 - resistant turnip ECD04-1, 5-8 - susceptible Chinese cabbage Ce-3

RAPD marker RA12-75a developed by Kuginuki et al. (1997) was used to survey resistant and susceptible parental lines of turnip and Chinese cabbage to determine whether this primer could be used for breeding of vegetable crops belonging to *B.rapa.* Results of the survey using RAPD marker RA12-75a did not show any polymorphism between clubroot resistant European turnip (ECD04-1) and susceptible Asian turnip (JRT1-1). The DNA polymorphism was detected between resistant turnip (ECD04-1) and Chinese cabbage line (Ce-3) (Fig. 1).

RAPD marker RA12-75$_{650}$ associated with the CR traits was amplified, extracted. Marker fragment was cloned and sequenced. GenBank (http://www.ncbi.nlm.nih.gov/GenBank) database searching analysis showed high homology with fragment of clone T24I21 chromosome 2 *Arabidopsis thaliana* and fragment of *Brassica napus* subsp. *napus* AtPP-A14 gene encoding putative S-adenosyl methionine salycilic acid carboxyl methionyl transferase and *slg* gene for 5-locus glycoprotein.

Specific primer pairs were synthesized on the basis of the sequence data (Tabl. 1). Nine combinations of primers were tested to amplify RA12-75$_{650}$ band.

Table 1

Specific primer pairs for RA12-75$_{650}$

Primer name	5'-3' sequence
>SCARp1F	GGA TCC ATT ATG CGG GCA GTT AG
>SCARp50F	CTA GTT AAA TGG CTC TGC GGT TG
>SCARp91F	GAG CTT GAT CTG CTG CCA TCG G
>SCARp406R	CAC GCG ATG AAT ATG ATC CTT AG
>SCARp463R	GGA TTG GGA GGT ATG TGG TAG AG
>SCARp636R	ATG CGG GCC AGC CAA TTA GGC

The PCR analysis using the primer pair

One or two bands were amplified using different combinations of primers used for detection of RA12-75$_{650}$. The PCR analysis using the primer pair SCARp91F-SCARp636R and SCARp50F-SCARp636R resulted in two clear codominant bands (a band for the susceptibility allele and a band for the resistance allele) (Fig. 2). However, the remaining 4 primer pairs amplified monomorphic or unclear polymorphic bands.

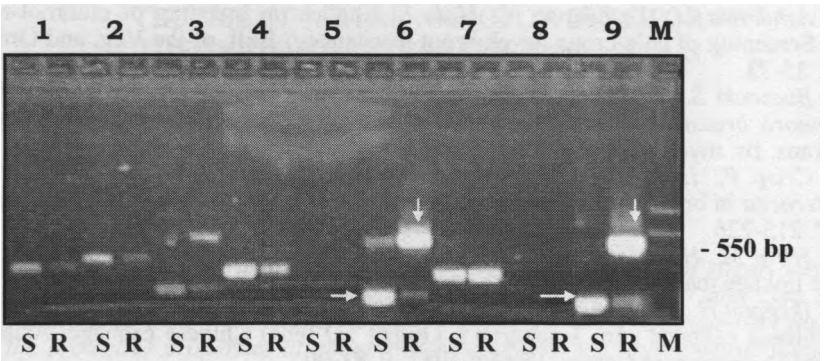

Fig. 2. DNA amplification using specific primer pairs S - sucseptible line Ce-3; R - resistante line ЕСД04-1. Lanes 1-9 combinations of primers: 1- SCARp1f-SCARp406r, 2- SCARp1f-SCARp463r, 3- SCARp1f-SCARp636r, 4- SCARp50f-SCARp406r, 5- SCARp50f-SCARp463r, 6- SCARp50f-SCARp636r, 7- SCARp91f-SCARp406r, 8- SCARp91f-SCARp463r, 9- SCARp91f-SCARp636r; markers associated with resistance allele are shown by vertical arrows, markers associated with susceptibility allele are shown by horizontal arrows

This specific amplification was further verified using segregating populations derived from a cross of turnip ECD04-1 and Chinese cabbage Ce-3 (Fig. 3). The result suggests that at least the marker amplified by specific primers SCARp91F-SCARp636R could be used for the breeding of CR Chinese cabbage cultivars.

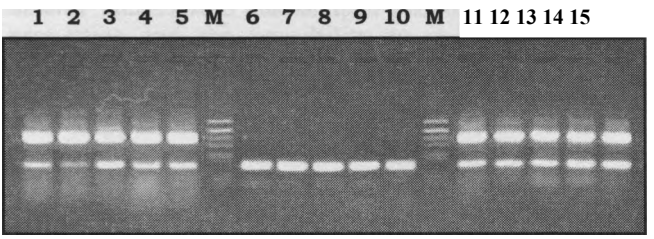

Fig. 3. DNA amplification using specific primer pairs SCARp91F-SCARp636R; Lanes: 1-5 - clubroot resistant turnip line ECD04-1; 6-10- susceptible Chinese cabbage line Ce-3; 11-15 - susceptible turnip line JRT1-1. M - molecular marker

The CR markers developed in the present study are codominant and PCR-based markers. Their polymorphisms are easily detectable with agarose gel electrophoresis. Thus, they will be an effective tool in marker-assisted pyramiding of CR genes in Chinese cabbage and finding other CR genes. However developed SCARp91F-SCARp636R marker is ineffective for population obtained from resistant and susceptible plants of turnip.

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