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Development and validation of CAPS and AFLP markers for white rust resistance gene in *Brassica juncea*

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Abstract White rust, caused by *Albugo candida*, is a very serious disease in crucifers. In Indian mustard (*Brassica juncea*), it can cause a yield loss to the extent of 89.9%. The locus *Ac2*(t) controlling resistance to white rust in BEC-144, an exotic accession of mustard, was mapped using RAPD markers. In the present study, we developed: (1) a more tightly linked marker for the white rust resistance gene, using AFLP in conjunction with bulk segregant analysis, and (2) a PCR-based cleaved amplified polymorphic sequence (CAPS) marker for the closely linked RAPD marker, $OPB06_{1000}$. The data obtained on 94 RILs revealed that the CAPS marker for $OPB06_{1000}$ and the AFLP marker E-ACC/M-CAA_{350} flank the *Ac2*(t) gene at 3.8 cM and 6.7 cM, respectively. Validation of the CAPS marker in two different F_2 populations of crosses Varuna × BEC-144 and Varuna × BEC-286 was also undertaken, which established its utility in marker-assisted selection (MAS) for white rust resistance. The use of both flanking markers in MAS would allow only 0.25% misclassification and thus provide greater efficiency to selection.

Introduction

White rust, caused by *Albugo candida* (Pers.) Kuntze, is an economically important disease of many crucifers. In Indian mustard (*Brassica juncea*), the available commercial varieties are highly susceptible to the white rust pathogen. It has been estimated that combined infection of leaf and inflorescence causes a yield loss to the extent of 62.7%, the loss being more severe (89.8%) as a result of staghead formation in susceptible cultivars (Lakra and Saharan 1989). Since the white rust pathogen is obligate in nature, getting sufficient inoculum for the timely inoculation of large segregating populations over generations is not feasible. Therefore, breeding for resistance against this pathogen has not been successful, although sources of resistance are available. The development and use of tightly linked DNA markers would enable indirect selection for the trait and therefore facilitate a directed transfer of the white rust resistance gene to agronomically superior genetic backgrounds.

In recent years, molecular markers have been used extensively to map and tag genes of agricultural importance in different crop species (Paterson 1996; Varshney et al. 2003). In the genus *Brassica*, molecular mapping of genes has been reported for several important traits, such as oil content, growth habit, fatty acid content, seed coat colour and resistance against diseases, including white rust (Quiros 2001; Lakshmikumaran et al. 2003). A locus (*ACA1*) controlling resistance to white rust has been mapped in *B. napus* (Ferreira et al. 1995) and *B. rapa* (Kole et al. 1996), using restriction fragment length polymorphism (RFLP) markers. A co-segregating RFLP marker has been identified for a gene (*Acr*) conferring resistance to *A. candida* in *B. juncea* (Cheung et al. 1998). Prabhu et al. (1998) mapped a resistance gene ($Ac2_t$) in *B. juncea* from a Russian source imparting resistance to a predominant Canadian isolate of *A. candida*. *B. juncea* accession BEC-144 from Poland shows resistance to the Indian isolates of the white rust pathogen. Earlier, we reported the identification of flanking markers for the locus controlling resistance to *A. candida* in this accession, employing random amplified polymorphic DNA (RAPD) markers and recombinant inbred lines (RILs; Mukherjee et al. 2001). However, the RFLP and RAPD markers identified for the target gene are not ideal for use in marker-assisted selection (MAS). RFLP markers demand more labour, time, DNA and also radioisotope. RAPD markers are not always reproducible in repeated assays and are usually dominant. The availability of tightly linked robust PCR-based markers is essential for the success of MAS. Reports on the actual use of markers

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in MAS, therefore, are limited (Cho et al. 1994; Hittalmi et al. 1995; Ribaut et al. 1997; Tanhuanpaa and Vilkki 1999).

It is possible to convert the available tightly linked RFLP markers into PCR-based sequence-tagged site (STS) markers (Oslon et al. 1989). Similarly, the critical RAPD markers can be converted into special STS markers named sequence-characterized amplified regions (SCARs; Paran and Michelmore 1993). Should the STS and SCAR markers fail to reveal any polymorphism, they can be easily converted to cleaved amplified polymorphic sequences (CAPS) by employing restriction enzyme digestion (Konieczny and Ausubel 1993). Unlike the RAPD marker, the CAPS is a PCR-based co-dominant marker that is reproducible and easier to manipulate in MAS (Caranta et al. 1999). There are reports where a CAPS marker has been used in constructing linkage maps in *Cryptomeria japonica* (Nikaido et al. 2000) and in the identification and comparative mapping of a dominant potyvirus resistance gene cluster in *Capsicum* (Grube et al. 2000). However, in the absence of tightly linked PCR-based markers, conscious efforts are needed to add new markers in the gene region. The use of bulked segregant analysis (BSA) in conjunction with a marker system such as amplified fragment length polymorphism (AFLP), which has a very high multiplex ratio, can aid rapid saturation of the target gene region with additional markers. AFLP is a rapid, powerful and reliable technique which is useful for DNA-fingerprinting, gene-tagging, high-density genome-mapping, MAS and positional gene-cloning (Becker et al. 1995; Thomas et al. 1995; Vos et al. 1995; Cervera et al. 1996; Hill et al. 1996;Qi et al. 1998; Xu et al. 1999; Xu and Korban 2000; Zhang and Stommel 2000). The aim of the present work was to develop a more tightly linked marker for the gene controlling resistance to white rust pathogen in BEC-144 (using AFLP markers) and to develop a SCAR/CAPS marker for the more closely linked RAPD marker reported by Mukherjee et al. (2001). Also, validation of the CAPS marker in different F_2 populations of *B. juncea* was conducted, which is critical before such markers can be recommended for MAS in conventional breeding programmes for white rust resistance.

Materials and methods

Plant materials

The plant materials used in the present study included a popular cultivar, 'Varuna' of Indian mustard, *B. juncea* L. (Czern and Coss), which is highly susceptible to white rust, a resistant exotic mustard collection BEC-144 and a set of 94 F_7 generation RILs obtained by selfing the F_2 progenies of the cross between Varuna and BEC-144. Bec-144, which is maintained at the National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute (IARI), New Delhi, by Dr. R.K. Katiyar, *Brassica* Breeder, is of Polish origin. For validation of the linked markers, F_2 mapping populations were derived by selfing the F_1 plants obtained from the crosses Varuna × BEC-144 and Varuna × BEC-286 (another resistant exotic mustard collection) in Phytotron, maintained at IARI. One of the cotyledons from each of the segregants was used for DNA isolation, as described by Prabhu et al. (1998). For carrying out BSA (Michelmore et al. 1991), equal amounts of DNA from ten highly resistant RILs were pooled to constitute the resistant bulk and, similarly, DNA from ten highly susceptible RILs was pooled to get the susceptible bulk.

Development and validation of CAPS marker

The polymorphic DNA bands showing linkage with the white rust resistance locus *Ac2*(t) in our previous work (Mukherjee et al. 2001) were amplified from the respective parents ($OPB06_{1000}$ from Varuna, $OPN01_{1000}$ from BEC-144) using the random primers OPB06 and OPN01 (Operon Technologies, Alameda, USA) in PCRs following the standard protocol of Williams et al. (1990). The amplified products were separated by electrophoresis on 1.5% agarose gels in 1× Tris-acetate EDTA (TAE) buffer for 4 h at 55 V. The critical bands ($OPB06_{1000}$, $OPN01_{1000}$) were cut individually from the gel, eluted using the QIA quick gel extraction kit (Qiagen, Germany) and cloned using PGEMT-Easy vector (Promega, Madison, USA). The white colonies growing on Luria agar plates containing 100 mg ampicillin/ml were picked and grown in Luria broth and the plasmid DNA was isolated using the Plasmid Miniprep kit (Bio-Rad, California, USA). The two critical fragments, $OPB06_{1000}$ and $OPN01_{1000}$, were then end-sequenced. Based on the sequence information, four specific sets of forward and reverse primers (22 nucleotides long) for both $OPB06_{1000}$ and $OPN01_{1000}$ markers were designed, synthesized (Promega, Madison, USA) and used to generate SCAR markers. In the case of the $OPN01_{1000}$ marker, all four primer pairs failed to amplify any fragment, whereas in the case of the $OPB06_{1000}$ marker, amplification of the expected 1,000 bp fragment was observed with the primer combination: forward 5′-gctcacttcagcaggggaaggc-3′ and reverse 5′-ggaaccgaacagacagacatgagttg-3′.

PCR amplification was performed in a total volume of 25 μl containing 30 ng of genomic DNA, 1× buffer (Bangalore Genei, India), 2.5 mM $MgCl_2$ (Bangalore Genei), 0.02 mM dNTPs (MBI Fermentas, Lithuania), 30 ng of each primer (forward, reverse) and 0.2 units of Taq DNA polymerase (Bangalore Genei). This specific primer combination led to the amplification of the expected 1,000-bp fragment in both Varuna and BEC-144, using the following PCR cycling conditions: 2 min 30 s at 94 °C, then 35 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C, followed by 10 min at 72 °C. Due to the failure of the SCAR marker to reveal any polymorphism between the two parental lines, cleavage of the amplified fragment was performed (after DNA precipitation using a Qiaex II kit; Qiagen, Germany) at 37 °C for 3 h, using four different restriction enzymes (*Hpa*II, *Mbo*I, *Alu*I, *Hae*III; MBI Fermentas, Lithuania) with the following restriction digestion conditions: 10 μl of PCR amplified product, 1.5 μl of 10× buffer and 1 unit of restriction enzyme in a final volume of 15 μl. The products were separated on 1.5% agarose gel in 1× TAE buffer. The linkage of the CAPS marker with the white rust resistance gene was confirmed by studying segregation in a set of 94 RILs obtained from the cross Varuna × BEC-144. For validation, the CAPS marker was used to screen F_2 mapping populations derived from the crosses Varuna × BEC-144 and Varuna × BEC-286.

AFLP analysis

AFLP procedures (Vos et al. 1995) were performed as described for the AFLP analysis system I (Life Technologies, USA), with the modification that each of the reaction components was halved. Fifty primer combinations were tried. The amplified products were separated by electrophoresis on 6% denaturing polyacrylamide gels containing 7.5 M urea in 1× Tris-borate EDTA buffer for 3 h at 65 V and 45 °C. A 20-bp DNA ladder (Life Technologies, USA) was used as a molecular marker. After electrophoresis, the gel was dried for 2 h and exposed to X-ray film (Bio MaxMR, Kodak) at −80 °C for 4–5 days.

White rust assay

Ninety-four plants from each of the crosses were grown in sterile soil under controlled conditions (25 °C temperature, 16:8 h photoperiod, 65% relative humidity). After about 8–10 days of seed germination, the plants were inoculated at the cotyledonary stage with *A. candida* spores. For *A. candida* inoculum preparation, spores of the fungus grown on susceptible parent Varuna were collected from the field. Freshly collected sporangia were added to 15 ml of distilled water in a 25-ml Erlenmeyer flask. The flask was covered with parafilm, mixed vigorously to suspend the sporangia and incubated for 2–3 h. At 12–16 °C, zoospores emerged and remained motile for 1–2 h. These were inoculated on Varuna. After 10–15 days of inoculation, infection appeared on the leaf surface. The spores were collected again and stored at −20 °C. Prior to inoculation, these stored spores were thawed and cultured under the conditions mentioned above to get motile zoospores. One of the cotyledons was inoculated with an inoculum load of 1.0×10^5 spores/ml. The inoculated seedlings were maintained for 24 h in the dark at day/night temperatures of 18 °C/15 °C and 100% relative humidity and thereafter at the conditions provided for seed germination. The infection appeared after 15 days of inoculation. The seedlings were phenotyped for resistance/susceptibility as described by Mukherjee et al. (2001).

Linkage analysis

Linkage relationships among the segregating loci were established using the computer package MAPMAKER/EXP. 3.0 (Lander et al. 1987). Map distances (in centiMorgans; cM) were derived using the Kosambi function (Kosambi 1944).

Results and discussion

Conversion of the RAPD marker $OPB06_{1000}$ into a CAPS marker

We earlier reported linkage of the RAPD markers $OPB06_{1000}$ and $OPN01_{1000}$ at distances of 5.5 cM and 9.9 cM on either side of the white rust resistance locus in BEC-144 that was designated as *Ac2*(t). These two critical fragments were cloned and end-sequenced. The PCR primers designed from the end sequence of the marker $OPN01_{1000}$ failed to amplify any fragment in either of the parents. This could be due to the presence of GC-rich (>70%) sequences, particularly around the forward primer-binding site. The intensity of the critical RAPD fragment $OPN01_{1000}$ was also low, indicating poor amplification of the region. In case of $OPB06_{1000}$, the expected 1,000-bp fragment was amplified with the specific primers in both the resistant and susceptible parents. When the amplified fragments from the parents were digested with four different four-cutter restriction enzymes, *Mbo*I revealed polymorphism between the parents. In the resistant accessions BEC-144 and BEC-286, the SCAR product was digested by this enzyme to give two fragments (800 bp, 200 bp), whereas in the susceptible variety Varuna, three fragments (650, 200, 150 bp) were generated (Fig. 1). In the susceptible RILs, a Varuna-like fragment pattern with the presence of 650, 200 and 150 bp fragments was observed. However, the 150-bp fragment was not clearly visible, while the 650-bp fragment was prominent. The 800-bp fragment was prominently visible in the resistant RILs similar to the resistant accessions. The CAPS marker for $OPB06_{1000}$ thus developed followed the expected Mendelian pattern of inheritance, giving a segregation ratio of 1:1 in 94 RILs obtained from the Varuna × BEC-144 cross. Based on linkage analysis, it was placed at 3.8 cM from the *Ac2*(t) gene (Fig. 2), which is comparatively closer than that of the corresponding RAPD marker $OPB06_{1000}$ (5.5 cM) reported earlier using the same set of the RILs (Mukherjee et al. 2001). This was because of the greater precision in genotyping the RILs using the CAPS marker that eliminated misclassification with the RAPD marker. The results clearly demonstrated the advantage of using a CAPS marker, as was reported earlier (Zheng et al. 1999), although it involved an additional step of restriction digestion of the PCR products.

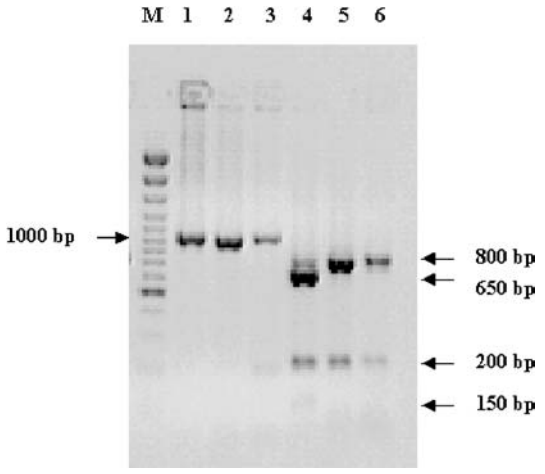

Fig. 1 Restriction analysis of the 1,000-bp SCAR product obtained with the RAPD primer OPB06 using *Mbo*I. *Lane M* 100-bp DNA ladder as size standard, *lanes 1–3* undigested SCAR products of Varuna, BEC-144 and BEC-286, respectively, *lanes 4–6 Mbo*I digests of the SCAR products of Varuna, BEC-144 and BEC-286, respectively. *Mbo*I generates 800-bp and 200-bp fragments in BEC-144 and BEC-286, whereas in Varuna three fragments (650, 200, 150 bp) are obtained

Validation of the CAPS marker

In the two F_2 populations obtained from the crosses Varuna × BEC-144 and Varuna × BEC-286, the plants carrying both the parent-specific fragments (650-bp fragment from Varuna, 800-bp fragment from BEC-144/BEC-286) were identified as heterozygotes and thus could be differentiated from the two homozygotic classes carrying either of the fragments. The CAPS marker segregated to give a genotypic ratio of 1:2:1 and the disease reaction followed the expected phenotypic ratio of 3:1. The segregation pattern in a set of ten resistant and ten susceptible F_2 plants from the Varuna × BEC-286 cross is shown in Fig. 3. As is evident from this figure, the CAPS marker distinguished homozygous resistant, heterozygous resistant and homozygous susceptible F_2 plants

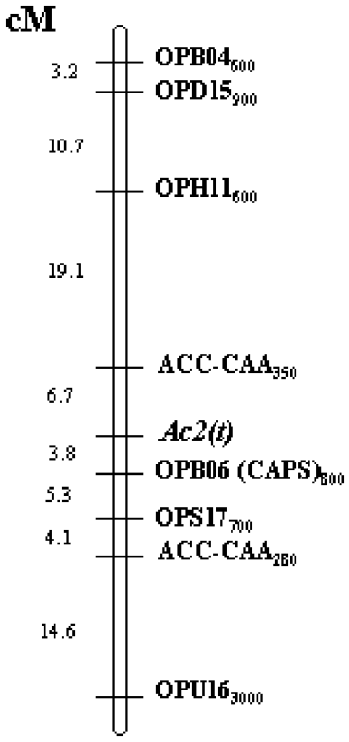

Fig. 2 Genetic map of the *Ac2*(t) gene region in mustard. The markers are indicated on the right side of the map. *OPB06 (CAPS)$_{800}$* is the CAPS marker, *ACC-CAA$_{350}$* and *ACC-CAA$_{280}$* are AFLP markers and the others are RAPD markers. The distances between two adjacent markers in centiMorgans (cM) are given on the left side of the map

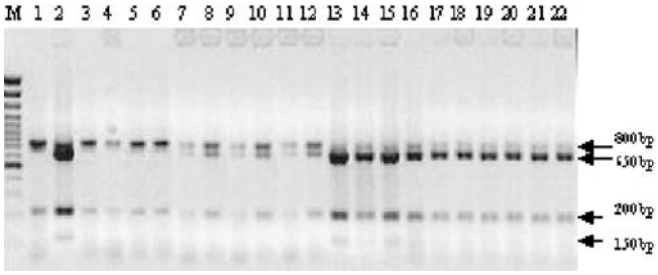

Fig. 3 Validation of the CAPS marker OPB06$_{1000}$ in the F_2 population of the cross Varuna × BEC-286. *Lane 1* BEC-286, *lane 2* Varuna, *lanes 3–6* homozygous resistant, *lanes 7–12* heterozygous resistant, *lanes 13–22* susceptible plants. *Lane M* 100-bp DNA ladder as size standard

from each other. In the F_2 population from the Varuna × BEC-144 cross, three individual plants showed a deviation in the phenotypes, compared with the genotypic data based on the CAPS marker, whereas in the Varuna × BEC-286 cross, four individual plants showed a deviation.

The CAPS marker for the white rust resistance gene developed in the present study thus identified homozygous resistant plants from homozygous susceptible and heterozygous plants in two different segregating populations. It can therefore be used successfully in the backcross transfer of white rust resistance from BEC-144 and BEC-286 to popular mustard varieties. These two accessions are highly effective against the virulent race(s)

Table 1 List of amplified fragment length polymorphism primers differentiating between parental and bulk DNA samples

*Eco*RI primers	*Mse*I primers	Number of bands polymorphic between BEC-144 and Varuna	Number of polymorphic bands differentiating the resistant bulk from the susceptible bulk
E-AAC	M-CAA	2	2
	M-CAG	4	–
E-AAG	M-CAA	13	3
	M-CAC	9	4
	M-CTT	6	4
E-ACA	M-CAG	7	–
	M-CTA	10	–
E-ACC	M-CAA	10	7
	M-CAG	6	–
	M-CTC	5	–
E-ACG	M-CAG	2	1
	M-CTA	4	–
	M-CTC	3	–
	M-CTG	4	–
E-ACT	M-CAA	8	–
	M-CAT	2	2
	M-CTC	5	–
	M-CTT	2	2
E-AGC	M-CAA	6	–
	M-CAC	5	1
	M-CAG	4	1
	M-CAT	14	–
	M-CTA	6	2
	M-CTG	2	–
	M-CTT	12	2
E-AGG	M-CAG	5	5
	M-CAT	5	4
Total	27	161	40

Fig. 4 Segregation pattern of the AFLP marker E-ACC/M-CAA$_{350}$ in a set of 28 recombinant inbred lines. *Lane M* 20-bp DNA ladder as size standard, *lane B* BEC-144, *lane V* Varuna. Disease reaction (*R* resistant, *S* susceptible) is indicated above each lane. The *arrowhead* shows the band of interest. The presence of the band corresponds to resistance and its absence to susceptibility

of the white rust pathogen *A. candida* prevalent in India and thus should serve as donors of resistance in mustard-breeding programmes. However, the presence of recombinants between the marker and the gene observed in the two F_2 populations revealed that 3–4% of the segregants would be misclassified based on the assay using this marker alone. Hence it was necessary to identify another tightly linked marker flanking the *Ac2*(t) gene.

Mapping of *Ac2*(t) using AFLP markers

Out of the 50 primer combinations tried, 27 differentiated the susceptible parent Varuna from the resistant accession BEC-144, giving 161 polymorphic fragments. Fourteen of these polymorphic primer combinations also differentiated the resistant and susceptible bulks from each other, giving a total of 40 informative markers (Table 1). Segregation of these 40 markers was carried out in 61 RILs obtained from the cross Varuna × BEC-144. One marker (350 bp in size) obtained with the primer combination E-ACC/M-CAA (Fig. 4) showed linkage with *Ac2*(t) at a map distance of 6.7 cM. This AFLP marker is closer to the *Ac2*(t) locus than the RAPD marker OPN01$_{1000}$ (9.9 cM) reported earlier. Now, on one side of *Ac2*(t), we have the CAPS marker for OPB06 at 3.8 cM and, on the other side, the AFLP marker E-ACC/M-CAA$_{350}$ at 6.7 cM (Fig. 2).

The AFLP marker was found linked to the white rust gene in the coupling phase and therefore would serve the same purpose as the coupling phase RAPD marker OPN01$_{1000}$ mapped earlier. Besides, genotyping with the AFLP marker would be more accurate than the RAPD marker, which gave poor amplification. However, in spite of trying 50 primer combinations, only one marker could be identified, which was more than 5 cM away from the gene. This necessitates different approaches to be employed to get closer to the target gene. A number of disease-resistant genes (R-genes) have been clo-ned and several others have been predicted in *Arabidopsis* (http://www.arabidopsis.org/; http://niblrrs.ucdavis.edu/At_Rgenes/Rgenes_Phylogeny/At_Rgenes_on_ Chromosomes.html). This information can now be utilized in mustard (which belongs to the same family, Cruciferae), employing a candidate gene-mapping approach. Such an approach has been successful in other crops (Yu et al. 1996; Yazaki et al. 2002). Primers can be designed using the available *Arabidopsis* sequences and specific R-genes can be amplified from the two parental lines of mustard. These primers are expected to work in mustard because of the conservation in gene sequences among different members of the Cruciferae (Quiros et al. 2001; Li and Quiros 2002; Parkin et al. 2002; Schranz et al. 2002). The observed co-linearity, particularly near the R-gene regions of *Arabidopsis* and *B. napus* as revealed by genetic mapping of R-EST sequences (Sillito et el. 2000), suggests the feasibility of the proposed approach. The polymorphic R-genes would be mapped along with the *Ac2*(t) gene using the same set of RILs. Suitable CAPS markers can also be developed for the non-polymorphic R-genes (Quint et al. 2002) to utilize all the available information in *Arabidopsis*. The results of the comparative mapping of the white rust resistance gene region involving *B. rapa*, *B. napus* and *Arabidopsis* (Kole et al. 2002) may help in targeting specific R-gene region(s) of *Arabidopsis*. Any co-segregating R-gene would be the most probable candidate for the white rust resistance gene of mustard. This would facilitate isolation of the *Ac2*(t) gene and, subsequently, the development of allele-specific markers based on the gene sequence.

In the present study, we could generate a co-dominant CAPS marker and identify an AFLP marker which flank the white rust resistance locus *Ac2*(t) present in accession BEC-144. There is only 0.25% misclassification when both are used in MAS. Conversion of the linked AFLP marker into a PCR-based SCAR/CAPS marker and its use along with the other flanking CAPS marker is expected to greatly facilitate rapid, large-scale genotyping required for MAS of white rust resistance both in backcross and in recombination breeding programmes in mustard.

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