



Allele mining in *Brassicas* screened for *A. brassicae* resistance

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The *Brassica* eponymously known as yellow beauty of cultivated crops has a wide range of agriculturally important species. Economically important *Brassicas* include oilseed, vegetable and condiment crops that are a valuable source of dietary fibre, vitamin C and other possible salubrious anticancerous compounds (Paterson *et al.* 2001). Cruciferous plants (*Brassicaceae*) are severely affected by *Alternaria* fungus and oleiferous crucifers being primary host for *Alternaria brassicae* cause severe economic losses in several ways (Humpherson-Jones & Maude 1982). *A. brassicae* is a necrotrophic pathogen that can infect any part of the plant and the symptoms include chlorotic and necrotic lesions on the leaf, petiole, stem, inflorescence, silique and seed (Kolte *et al.* 1987, Verma and Saharan 1994). Potential sources of resistance to *Alternaria* blight have not been reported but sources of tolerance in wild relatives such as *Sinapis alba*, *Camelina sativa*, *Erucastrum gallicum* and *Diplotaxis* species (Conn *et al.* 1988) has been reported. Identification and exploration of novel alleles existing at known disease responsive loci like NPR1 has been an unexplored area of research in *Brassica*. The NPR1 gene plays a pivotal role in conferring broad spectrum resistance to plants and also the wild alleles can be potential source of resistance. In the present study we describe the screening technique for *alternaria* and allelic variation of NPR-1 that exist at the disease responsive gene loci in different species of *Brassica*.

Pathogen isolation and identification: The pure culture of *Alternaria brassicae* was isolated from the field infected necrotic lesions of leaves. The leaves of infected plants were washed with sterile distilled water and cut into small pieces of 2 cm² area. These pieces were then placed on to the Potato Dextrose Agar (PDA) medium and incubated at 25°C under 12 h dark followed by 12 h light cycles for 7 days. Regular serial transfer of the culture obtained into fresh PDA plates resulted in obtaining pure culture of the isolate. The isolated pathogen was identified as *Alternaria brassicae* based on the conidial and colony morphology and its pathogenicity was confirmed following Koch's postulates.

Plant material and growth: The seeds of *Brassica* species and their wild relatives were grown in 20 cm earthen pots with autoclaved soil and FYM in the ratio 1:1 with replicates in net house during the season (October – February). The plants were grown up to 45 days and then used for pathogen inoculation.

Pathogenicity test: Pathogenicity was tested by inoculation of leaves using pathogen spore suspension with a density of 6×10^3 spores/ml. The plants were covered by polythene bag under wet condition. Humidity of 90% was created by keeping the polybag into incubation chambers. Dark conditions were provided for the initial 12 hr after inoculation. After 12 hr the 16 hrs light and 8 hr dark conditions are given alternatively. The observations were taken upto 72 hr post inoculation.

Ratings: In order to measure the reaction of different plant species to *Alternaria brassicae* infection, two parameters namely incubation period and yellowing of the leaf area were considered. Incubation time intervals taken for observation were 1, 6, 12, 24, 48 and 72 hr post inoculation. When no symptoms developed a score of 0 was assigned and a maximum score of 10 was allocated for yellowing beyond 3.0 cm (Table 1). The general principle of higher the score, higher the susceptibility and lower the score higher the resistance was employed to evaluate the response of the species.

NPR1 gene isolation: Genomic DNA was isolated from young leaves of different species using the CTAB method of DNA isolation. The DNA quality and quantity was checked using nanodrop. The DNA was used for PCR with NPR1 gene specific primers. The gene specific primers were designed for the consensus sequences obtained by MSA (Multiple sequence alignment using Bioedit) of the available sequence information of NPR1 from different species using Primer3 and oligoanalyzer softwares. The NPR1 gene was amplified using the PCR program set at 95°C for 5 min as initial denaturation followed by 95°C for 1 min, 56°C for 30s, 72°C for 1 min and 72°C for 10 min for 35 cycles. The PCR products were run on 1.2% agarose gels and eluted using Genetix elution kit. The purified PCR products were cloned into pJET vector (Fermentas) by blunt end cloning. The clones were screened for presence of genes

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Table 1 Relative scores for disease parameter of cultivated and wild allied species of Brassica exposed to *Alternaria brassicae*

Species	Infection score based on zone of yellowing	Diameter of the infection zone (cm)
Cultivated		
<i>Brassica carinata</i>	8	4
<i>Brassica juncea</i>	10	8
<i>Brassica napus</i>	2	0.25
<i>Brassica nigra</i>	6	0.50
<i>Brassica oleracea</i>	1	0.10
<i>Brassica rapa</i>	4	0.75
Wild		
<i>Brassica caudatus</i>	8	1.0
<i>Brassica spinescence</i>	7	1.5
<i>Diplotaxis muralis</i>	2	0.0
<i>Diplotaxis erucoides</i>	0	0.0
<i>Eruca sativa</i>	5	0.10
<i>Erucastrum gallicum</i>	4	0.25
<i>Erucastrum canariense</i>	2	0.0
<i>Sinapis alba</i>	1	0.0

using PCR and restriction digestion. The confirmed clones were sequenced by outsourcing. Some of the purified PCR products were directly used for PCR sequencing.

Phylogenetic analysis: The NBS-LRR-TIR domains (amino-acids) of different species of Brassica were taken along with other plants like *Arabidopsis*, sugar beet etc for deciphering the phylogeny. The gene and the corresponding protein sequences obtained from the sequencing results and

databases were brought together in FASTA format. The sequences were then aligned using ClustalW followed by phylogenetic tree construction using Clustal W2-phylogeny software available at EMBL-EBI website. Conserved domains database was used to determine the conserved domains existing in the sequences.

Pathogen identification and isolation: The pathogen *Alternaria brassicae* was isolated from infected leaf samples collected in *Brassica* growing belt. The humid conditions were congenial for development of *Alternaria* spores on host tissue. Microscopic examination followed by continuous sub-culturing led to isolation of pure culture of *Alternaria brassicae*. The *Alternaria brassicae* conidia were elongated muriform, beaked, bottle shaped and septate and were found to have 6-10 transverse septa oblong body and a terminal beak resembling the genus *Alternaria*. This result is in accordance with those described by Giri *et al.* (2013) who have studied the *Alternaria brassicae* microscopically.

In vivo infection and screening: In-vivo infection of plants with isolated *Alternaria* spore produced a differential disease development response. Considerable variation was observed among the different species. *Brassica juncea* and *Brassica carinata* were found to be most susceptible giving an early disease response showing leaf discoloration at 3 DAI. The leaf infection lesion is also largest of 8 and 4 cm respectively for *B. juncea* and *B. carinata*. *B. nigra* and *B. rapa* have developed yellow coloration at 5 DAI. *B. oleracea* followed by *B. napus* were found to be tolerant to infection by *Alternaria brassicae*. This is in accordance to the results investigated by Dubeck and Degenhardt (1975). The thick waxy layer on the leaf surface of these two species would act as barrier for hyphal penetration thus giving a delayed infection response. Among the wild species, *B. spinescence* and *B. caudatus* have been very susceptible to *Alternaria* infection. Prominent chlorotic zones were observed in the

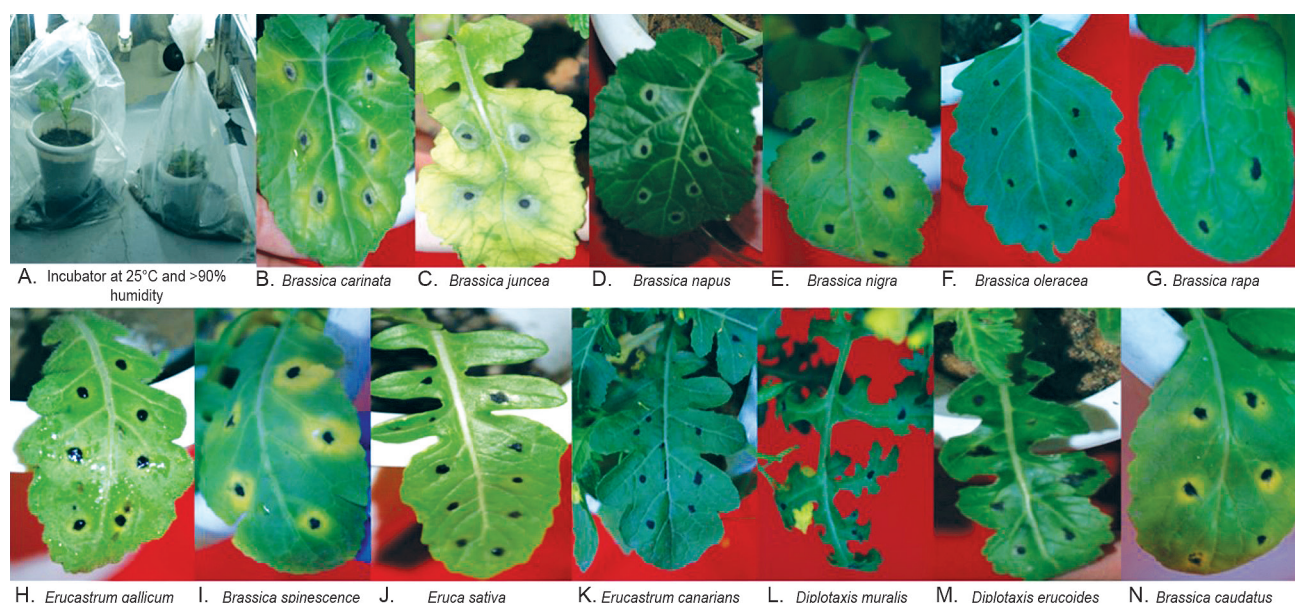


Fig 1 Screening of brassica wild and cultivated genotypes at 25°C and 90% RH; B-N, different genotypes showing variable symptoms in response to *Alternaria* infection.

susceptible species and the diameter of the chlorotic zone (Table 1) showed the severity of infection in different species (Fig 1). According to Sharma *et al.* (2002) wide variations in the incubation periods of all the species have been observed. The results of *B. juncea* are in accordance with the previously reported results but not for *B. carinata* which was earlier shown to be resistant. The wild relatives of *Brassica* species, i.e. *B. spinescence* was in accordance with previous results but for *B. caudatus* whose resistance response has not been recorded.

A wide variation was observed in reference to incubation period for the development of visible symptoms. Some species of *Brassica* including wild and cultivated developed visible symptoms as early as 48 hr and the wild species like *Eruca sativa*, *Erucastrum gallicum* showed symptoms at 72 hpi. The *Alternaria* leaf spot resistant wild species like *Erucastrum canariense*, *Sinapis alba*, *Diplotaxis muralis*, *Diplotaxis eruroides* did not develop any symptom until 98 hpi. However, *Diplotaxis muralis* developed some yellowing after 5 days post inoculation and the remaining three species showed significant resistance to *Alternaria* leaf spot. This wide variation in the incubation period was observed among the species of *Brassica* and *Diplotaxis* genus and the time period varied from 3 to 15 days post infection (Sharma *et al.* 2002). Evaluation under artificial conditions has been essential as growing the wild and cultivated in open fields may render them to become competitive weeds and may also disturb the genome of cultivated species (Sharma *et al.* 2002). The leaf spot with yellowing symptoms were clearly visible showing that the experimental set up was conducive for the pathogen to infect. Also under natural conditions the spore concentration may not be uniform and there may be escapes. This scenario can be overcome by artificial infections with spore counted inoculums.

Cloning and sequencing of NPR1 alleles: The NPR 1 gene which has been shown to play very important role in disease resistance was isolated from the cultivated and wild species of *Brassica* using gene specific primers designed at UTR regions and also spanning the gene sequence at every 300 bp interval. Shi *et al.* (2010) have isolated the NPR 1 gene in *Cacao* using degenerate PCR based on alignment of NPR1 homologs from *Arabidopsis*, *Brassica* and *Carica papaya*. About 1.9 kb alleles of NPR1 genes were isolated from different species of *Brassica* and these represented presence of 5 exons in the full length genes as identified by FGENESH. The sequences contain BTB domain as analysed by PROSITE database which act as a transcription factor domain. These domains are crucial components of NPR1 and provide functions relating to NPR1-dependent co-activation of TGA transcription factors and protein-protein binding (Rochon *et al.* 2006). The nucleotide additions and deletions were observed in the BTB domain region. Further the multiple sequence alignment of the gene sequences belonging to wild and cultivated *brassica* showed a distinct evolutionary branching as represented in the phylogenetic tree. The bootstrap values above 60 represent a close association of the gene sequences among

the different species. The resistant wild alleles fall into one clad and the susceptible alleles into another. The NPR1 genes from different crop species were also aligned with the isolated *Brassica* genes and a cladogram of the same represented close association between the sequences with distinctness along each clad (Peraza-Echeverria *et al.* 2012). Multiple sequence alignment of all the species at nucleotide and species level identified conserved domains in each of the proteins. The domains BTB, ankyrin repeat and NPR1 like were found in the sequences analysed by EMBL domain identifier. An upstream promoter region was isolated from the *Brassica juncea* genome and the analysis by plant care software represented the presence of important promoter motifs such as TATAAT, CAT and GC box in addition to a TCA cis-element for salicylic acid responsiveness.

SUMMARY

The non-expressor of pathogenesis related gene 1 (NPR1) has been an important component of the SA/JA mediated mechanism of defence in plants. *Brassicas* have been major group of crop plants that are facing huge yield losses due to biotic stresses especially through *Alternaria* blight caused by *Alternaria brassicae*. Among the plethora of proteins, the NPR1 protein coding gene has been emphasised upon and an attempt has been made to isolate NPR1 alleles from different *brassica* species. The sequences were annotated using FGENESH and a maximum-likelihood tree was constructed using NPR1 genes from cultivated and wild *brassica* and also NPR1 from other crops. Understanding the genome structure of NPR1 and tagging the resistance alleles to the genomic regions of NPR1 among all species of *Brassica* has been aimed at in the present study.

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