Induction of defense genes in *Brassica juncea* against *Alternaria brassicae*

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Abstract

Brassica juncea is the second most important oil crop in India, next to groundnut only. But unfortunately, up to 35-42% yield losses are reported each year because of fungal disease caused by *Alternaria brassicae.* The absence of sources of resistance against this pathogen among sexually compatible relatives emphasizes the need of adopting biotechnological strategies to improve the resistance of *B. juncea*. Therefore our project is aimed at isolating and characterizing important defense-associated genes in this species and study their regulation in order to generate fungus-resistant *Brassica.* Here, we report on the differential regulation of different antifungal genes, namely, PR1, PR2 (glucanase), PR3 (chitinase), PR4, PR5 and defensin by distinct signaling pathways that are mediated by salicylic acid (SA) or jasmonic acid (JA) in *B. juncea.* We shall also present our work in producing defense-induced cDNA libraries in *B. juncea* and the isolation of above mentioned defense genes and their characterization. In addition, we have also isolated a regulatory defense gene NPR1 which is an activator of transcriptional factors. Its characterization and relevance will be discussed. We have also isolated a gene coding a protein that interacts with the R-gene product. This gene product probably plays significant roles in the infection signal transduction pathway(s).

Key words: *Brassica juncea, Alternaria,* fungal infections, defense genes, transgenics

Introduction

Vegetable edible oil plays an important role in human nutrition and economy of India. *Brassica juncea* is the second most important oil seed crop in India next to groundnut. Average acreage for this crop in India is 6.1 mha, the average yield per hectare is very low (780 kg/ ha) when compared to world average of 1300kg/ha. The major factors for poor yield of oil seed crop *Brassica* are insect and diseases. Among various fungal diseases *Alternaria* blight caused by *Alteranaria brassicae* is devastating and can cause yield losses of up to 30–40%. There is no source of resistance against this pathogen among the sexually compatible relatives of *Brassica* thereby emphasizing the need of adopting biotechnological strategies to improve resistance (Tewari & Mithen 1999). Little is known about defense mechanisms of *B. juncea* (Zhao & Chye 1999, Grover & Gowthaman 2003). To improve the resistance in Brassica we need to know what are the genes induced during infection, how they are regulated and what are their function. It is very important to isolate defense genes and cDNA clones from the same species because these regulatory genes tend to be crop specific. Since regulatory genes like NPR1, PAD4, work upstream in signal transduction and can induce a whole battery of antifungal genes to combat the incoming pathogen, the identification of such genes will have direct practical applications in developing fungus resistant transgenic plants (Glazebrook 2001, Rommens & Kishore 2001). Further to employ these genes for improving resistance, we should have knowledge of how they are regulated endogenously in the plant. Therefore, to meet these objectives, cDNA libraries of *Brassica juncea* were made under control conditions and after treatments with known inducers such as salicylic acid and jasmonic acid (Clarke et al., 2000). Defense-induced antifungal genes (eg., PR protein genes) and regulatory genes (eg., NPR1) were isolated from these libraries and their regulation was studied.

Material and Methods

Growth of Brassica plants and treatments with SA and JA: B. juncea (var RLM198) plants were grown in growth chamber at 22- 24^0 C under a 12 h light and 12 h dark at 80% humidity. Three week old plants were sprayed with water, SA (5mM) or JA (100µM). Leaf samples were harvested at 0, 2, 4, 8, 12, 24, 48 and 72 h after spraying.

Studying regulation of defense genes: RNA was isolated from the above samples. We studied the induction of PR1, PR2 (glucanase), PR3 (chitinase), PR4, PR5, PDF, NPR1 and PAD4. NPR1 and PAD4 are early regulatory genes while PR1-5, PDF are late, actually executing antifungal genes. Induction studies were done by Northern using the corresponding gene probes. The probes of these 8 genes were made using available *Arabidopsis* sequence. For all the 8 probes, primers were chosen in the conserved (among different species) regions and which does not contain any intron. Using these primers, probes were synthesized by PCR using *Arabidopsis* DNA as template. Length of the probes was from 300 to 900 bp. The PCR products were cloned in pGEMTEasy vector (from Promega). Sequence of each probe was confirmed and then used for doing Northern studies. For RNA isolation, probe making, northerns, PCR and cloning, methods from Sambrook et al. (1989) were used.

Construction of cDNA libraries: cDNA libraries were made from poly(A) RNA of control (treated with water), SA and JA treated plants using SMART cDNA library construction kit of BD Biosciences (catalogue no. K1051-1). The cDNA is in Sfi1 A & B sites of lambdaTriplEx2 in this library.

Screening of cDNA libraries for defense genes: For screening, the unamplified libraries were used. JA induced library was used for screening chitinase clone and SA-induced library was used for screening of PR1, glucanase, NPR1 and PAD4. Again, method from Sambrook et al. (1989) was used.

Rescuing the positive clones and sequencing: cDNAs from the positive clones were rescued in the plasmid form in *E. coli* BM25.8 background (provided in kit from BD and has Cre recombinase activity). Plasmids were machine-sequenced using sequencing- primers provided in the kit.

Results

Regulation of defense genes: We studied the induction of PR1, PR2 (glucanase), PR3 (chitinase), PR4, PR5, PDF, NPR1 and PAD4. NPR1 and PAD4 are early regulatory genes while PR1-5, PDF are late, actually executing antifungal genes. The probes of these 8 genes were made using available sequence of their homologs in other species. For all the 8 probes, primers were chosen in the conserved (among different species) coding regions. Using these primers, probes were synthesized by PCR using Arabidopsis DNA as template. Length of the probes was from 300 to 900 bp (Fig.1).

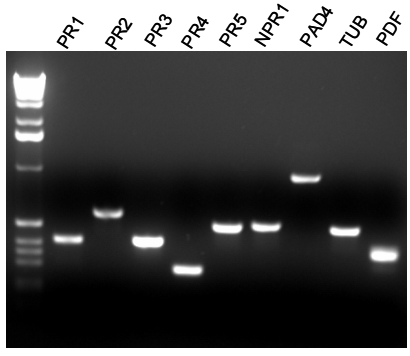

Fig. 1. Synthesis of probes by PCR

The PCR products were cloned in pGEMTEasy vector. Sequence of each probe was confirmed and then used for doing Northern studies. Interestingly, different defense genes were induced by different signaling pathways e.g. chitinase (PR3) is induced by JA treatment and not by SA (Fig. 2C) while PR1 and PR2 (glucanase) are induced many fold by SA (Fig. 2A, B). Although chitinase and glucanase seem to be co-induced and work together for hydrolyzing fungal cell wall, they are induced by different signaling pathways. NPR1 and PAD4 *Arabidopsis* probes did not work for studying corresponding *Brassica* genes. Probably the induction is not strong to be noted by heterologous probe

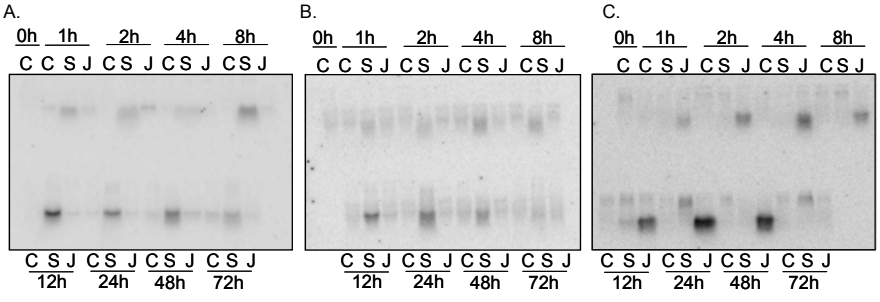

Fig. 2. Regulation of PR1, PR2 (glucanase) and PR3 (chitinase) transcripts.

Construction of cDNA libraries: Northern studies indicated that RNA from leaves harvested at 12 h after SA and 24 h after JA treatment was appropriate to make cDNA libraries for isolation of defense gene cDNA clones. cDNA libraries were made from poly(A) RNA of control (treated with water), SA and JA treated plants. All the amplified libraries have 10^9 to 10^{10} pfu/ml and have been stored at 4°C, and also at –70°C.

Screening of cDNA libraries for defense genes: For screening, the unamplified libraries were used. JA induced library was used for screening chitinase clone and SA-induced library was used for screening of PR1, glucanase, NPR1 and PAD4. After primary and secondary screenings, 24 putative positive clones were obtained for each PR1, PR2, PR3, NPR1 and PAD4.

Rescuing positive clones and sequencing: cDNAs from the positive clones were rescued in the plasmid form in *E. coli* BM25.8 background and were machine-sequenced. Different genes which have been isolated and sequenced (some of them have already been submitted to NCBI) are listed in Table 1.

Table 1. Defense gene cDNAs isolated and their accession numbers

Name of the gene	Gene Bank accession number
Glucanase-1	DQ359125
Glucanase-2	DQ359126
Chitinase	DQ359127
PR1	DQ359128
NPR1	DQ359129
RIN (Resistance gene Interacting Protein) homolog	EF165002

Discussion

Regulation study of pathogenesis-related (PR) protein genes by SA and JA indicate very clearly that PR1 and PR2 (glucanase) genes are induced by SA and not by JA while PR3 (chitinase) gene is induced by JA and not by SA. Similar induction pattern was also reported in *Arabidopsis* (Thomma et al., 1998). These data would indicate the existence of at least two separate hormone-dependent defense pathways in *Arabidopsis* and *Brassica* that contribute to resistance against distinct microbial pathogens and it has been conserved in these two species. Nevertheless, it is interesting to note that chitinase and glucanase work synergistically towards inhibition of fungal growth (Mauch et al., 1988) in spite of being induced by different signaling pathways.

Several positive clones screened from SA- and JA-induced cDNA libraries were sequenced to get those which were full length cDNAs having start and stop codons and also in some cases 5' and 3' untranslated regions of the cDNA. PR1, glucanase, chitinase and NPR1 cDNAs sequences have already been submitted to NCBI and they bear 76.2 %, 74.6%, 77.1% and 63.3% respectively to *Arabidopsis* corresponding cDNAs. All these are full length cDNAs except chitinase which is being made full length by RACE. Besides these, we have also obtained partial length clones of some more defense genes like thionin, polygalacturonase and glutathione-transferase which have to be sequenced further. By over- expressing, cDNAs of these antifungal genes are being used for the production of fungus-resistant transgenic *Brassica.* We have also identified a cDNA that shows similarity to a *Arabidopsis* resistance gene-interacting (RIN) protein, and are currently.in the process of identifying proteins interacting with RIN in order to characterize of upstream regulatory proteins.

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