Caspase-3 like Protein in Wheat-\textit{Tilletia indica} Dual Culture System as Potential Biomarker for Host Resistance to Karnal Bunt

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Immuno-histochemical staining of dual co-cultured calli of wheat-\textit{Tilletia indica} having resistant and susceptible line using anti-mycelial and anti-teliospore polyclonal antibodies revealed the differential resistance response as indicated by pathogen invasion, mycelial colonization, plasmolysis and immunolocalization of chlamydospore like structures. The p$^{53}$ and caspases, crucial proteins of cell death pathways were detected by immunological procedures using heterologous immunoprobes in fungal colonized wheat calli. There was no difference in p$^{53}$ expression in resistant and susceptible lines at different days after inoculation (DAI) of \textit{T. indica} on calli raised from mature embryos. The expression of p$^{53}$ started 14 DAI, increased steadily up to 21 DAI and became constant at 30 DAI showing the saturation of expression level. Studies on the expression of caspases revealed the absence of caspase-1 like protein at different stages of growth. The caspase-3 like protein was induced at 3 DAI in both hosts and its expression steadily increased at 14 DAI and reached a plateau at 21 DAI. A remarkable difference in the level of expression of this protein between resistant and susceptible co-cultured calli was observed. Differential activation of caspase-3 like protein was observed in 10 hosts and non-hosts. It was found to be correlated with the degree of susceptibility and the expression of resistance at cellular level. Hence, it could be used as a potential biomarker for identification of Karnal Bunt resistance sources.

Keywords: wheat, dual culture, sporidia, Karnal bunt, \textit{Tilletia indica}, resistance, bio-marker, caspase-3, p$^{53}$, immunoassay

Introduction

Karnal bunt (KB), caused by \textit{Tilletia indica} (syn. \textit{Neovossia indica}) is an economically important disease of wheat. The epidemic occurrence of the disease in India and outside has serious consequences like poor grain quality and reduced yield. Despite all efforts made to control the disease through cultural and chemical treatment methods, scientists have limited success in achieving the complete control. Due to complex nature of the KB pathogen, screening of wheat germplasms for identification of resistant cultivars and their use has become important for successful management of disease (Gill \textit{et al}, 1993; Hoffman, 1983). However, screening of wheat varieties, although they differ in their degree of susceptibility to KB pathogen under field conditions, is difficult due to experimental, agricultural and natural constraints. Moreover, conventional screening methods for identification of the source of resistance to KB in wheat, under natural conditions, were found unreliable and inadequate. Hence, it is important to develop an effective screening tool for the identification of wheat sources with resistance to KB.

\textit{In vitro} selection of plant tissue for disease resistance is based on either the inability of the callus, cells or protoplasts to support growth of the pathogen or the ability of the host callus, cells or protoplast to withstand pathogen’s toxins (Daub, 1986). These methods, which have also been attempted for \textit{in vitro} screening of wheat varieties for KE resistance, however, failed to differentiate resistant and susceptible wheat cultivars particularly in dual culture system (Daub, 1986; Singh \& Singh, 1989; 1992; Gill \textit{et al}, 1993). It was due to lack of precise molecular and genetic changes, which occur as a result of pathogen infection. Therefore, a need was felt to evaluate new panels of biomarker(s) as a screening aid to \textit{in vitro} selection for the resistance source.

Programmed cell death (PCD) functions in many aspects of animal and plant development and in their response to stresses (Greenberg, 1996; Levine \textit{et al}, 1996; Martins \& Earnshaw, 1997; Mc-Conkey \& Orrenius, 1994; Stewart, 1994). It is employed in both fungal pathogenesis and plants’ defence against...
fungal pathogens (Greenberg, 1996; Levine et al., 1996). Although a detailed mechanism of PCD is still largely unknown, recent studies suggest that cell death pathways in plants are morphologically and biochemically similar to those of animals (Greenberg, 1996; Levine, et al., 1996; Wang et al., 1996). This further suggests that genes and proteins involved in PCD have been highly conserved across the kingdoms. The cell death proteins viz. p53 and cascade of caspases have emerged as the key proteins in the regulation of cell death pathways in animals (apoptosis) and plants (PCD). The activation of cysteine protease (called caspase), as observed in plants in response to pathogen trigger suggests these enzymes are components of an essential, conserved cell death mechanisms (Solomon et al., 1999).

In the present study, attempts were made to develop dual culture system of wheat-Tilletia indica as a novel in vitro screening aid for the host and non-host cultivars for KB resistance. The dual culture system was also employed as a model to investigate the effect of fungal colonization on programmed cell death of hosts and non-hosts. Expression of conserved cell death proteins e.g. caspases, p53, was taken as an index for PCD. The potentiality of these cell death proteins to serve as novel biomarkers for delineating the KB resistance was also evaluated.

Materials and Methods

Collection of Seeds and Pathogen

Eight cultivars of wheat having different degree of resistance to KB and one cultivar each of barley and rice were collected from Crop Research Centre, G B Pant University of Agriculture and Technology, Pantnagar; and Department of Genetics and Plant Breeding, Punjab Agricultural University, Ludhiana respectively. The degree of resistance to KB of these wheat cultivars and non-hosts is given in Table 1. The virulent strain of Tilletia indica (Pantnagar isolate) was collected from Department of Plant Pathology, G B Pant University of Agriculture and Technology.

Induction of calli

The seeds of all cultivars were surface sterilized with 0.1% HgCl₂ for 5 min and 0.5% sodium hypochlorite followed by washing with sterile distilled water (5 min × 3). These were soaked for two days in sterile distilled water. The embryos were dissected with the help of scalpel and forceps, teased and placed in MS basal medium supplemented with 2 ppm 2, 4-D and the cultures were placed in incubation chamber in dark at 22±1°C. After six weeks of growth with sub culturing at 25 days of interval, the calli were transferred in maintenance medium containing MS basal medium, supplemented with 10 ppm (10 mg/l) proline for long-term maintenance.

Establishment of Dual Culture

The dual culture of wheat-T. indica, barley-T. indica, and rice-T. indica, each with appropriate controls were established by adding 200 sporadia on each callus, which was maintained in maintenance medium with 20 ppm fungicide (Captan 75 wp). The plates were then incubated at 22±1°C in incubation chamber with alternative light and dark cycles of 12 hrs each for 30 days.

Production of Polyclonal Antibodies

New Zealand white albino rabbits were used for production of antisera against partially purified mycelial antigens as described earlier (Varshney, 1999). The partially purified antigenic preparation was taken and emulsified with an equal volume of Freund’s complete adjuvant before injection. The antigen was injected in two female rabbits. About 0.5 ml emulsified antigen(s) per rabbit containing 500 μg of mycelial antigen was administered through subcutaneous mode, intra-peritonial mode and footpad at multiple sites. The antigen emulsified in equal volume of Freund’s incomplete adjuvant was used for booster injections and given at intervals of 10, 20 and 30 days after primary immunization. One week after the last injection the rabbits were bled from a marginal ear vein. The clear serum was decanted from the clotted blood into test tubes, centrifuged at 5000 rpm for 5 min to get rid off any

<table>
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<tr>
<th>Cultivar/Variety</th>
<th>Group</th>
<th>Susceptible/Resistant</th>
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<tr>
<td>HD 29</td>
<td>Aestivum</td>
<td>Resistant</td>
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<tr>
<td>HD 30</td>
<td>Aestivum</td>
<td>Resistant</td>
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<td>PDW 215</td>
<td>Durum</td>
<td>Resistant</td>
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<tr>
<td>PBW 343</td>
<td>Aestivum</td>
<td>Moderately susceptible</td>
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<td>UP 2338</td>
<td>Aestivum</td>
<td>Moderately susceptible</td>
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<td>WH 542</td>
<td>Aestivum</td>
<td>Highly susceptible</td>
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<td>HP 2329</td>
<td>Aestivum</td>
<td>Highly susceptible</td>
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<tr>
<td>Raj 1535</td>
<td>Aestivum</td>
<td>Highly susceptible</td>
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<tr>
<td>HBL 371</td>
<td>Barley</td>
<td>Non-host for KB</td>
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<tr>
<td>(Hordem vulgare)</td>
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<tr>
<td>4036</td>
<td>Dehraduni Basmati</td>
<td>Non-host for KB (Oryza sativa)</td>
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remaining cells. The antiserum was finally frozen at -20°C.

The detection and quantification of teliospores in dual culture calli were done using polyclonal antibodies raised against teliospores in (Kumar et al, 1997). The DIBA and microtitre ELISA were developed for immunodetection and quantitation of fungal mycelia and teliospores as described earlier employing 1:1000 ratio of anti-mycelial and anti-teliospore antibodies and goat-anti rabbit IgG conjugated with alkaline phosphate (Rai et al, 1998).

**Immuno-histochemical Localization of Fungal Pathogen**

The localization of fungal mycelium and teliospores in dual culture of calli was performed by fixation and embedding of the callus tissues. The embedded tissues were placed in digital microtome and sectioned at 10-20 μm. The sections were immunoprocessed using anti-mycelial antibodies (1:250) against purified mycelial antigen and anti-teliospore antibodies (1:100) separately. The fungal mycelia and teliospores were visualized in callus tissues using goat anti-rabbit immunoglobulins conjugated with colloidal gold (1:50) and observed under light microscope.

**Extraction of Total Proteins**

Total proteins were extracted from calli of hosts and non-hosts as described earlier with slight modification (Fieler et al, 1990). The overnight frozen callus was ground with minimum volume of extraction buffer and a few pieces of cover slips to break the cell wall more efficiently. The ground mixture was centrifuged at 19,600-x g for 15 min at 4°C without filtration. The precipitation of proteins with acetone was not necessary. The protein was estimated by dye binding method (Bradford, 1976).

**Immuno-detection of Cell Death Proteins**

The expression of p53 and caspases (caspase-1 and caspase-3) dual cultured wheat calli was detected by performing dot ELISA and microtitre ELISA.

**Dot Immunoblot Binding Assay (DIBA)**

The protein samples prepared as described earlier were spotted on nitrocellulose membrane strip and allowed to dry. The nitrocellulose strip was blocked by immersion in 2% bovine serum albumin for 1 hr at room temperature for non-specific protein binding. The membrane was then rinsed with fresh changes of PBST for 2 min, then 10 min and finally with 5 min agitation. The membrane was then probed with either of polyclonal antibodies i.e. rabbit anti-human p53 antibodies gifted by Dr Dover Fattori of Scotland Antibody Production Unit (SAPU), UK, or rabbit anti-human caspase-1/caspase-3 procured from Santa Cruz Biotechnology Inc, USA and subsequently processed for development of dots using goat anti-rabbit secondary antibodies conjugated with alkaline phosphate and a precipitable substrate BCIP/NBT (M/s Bangalore Genei, India).

**Microtitre ELISA**

The extracted protein from calli was coated on 96-well microtitre ELISA plate (Corning) overnight at room temperature. The wells were incubated with 2% BSA for 30 min for non-specific blocking, rinsed thrice in PBS and then incubated at room temperature for 1 hr with 1:500 dilution of rabbit polyclonal anti-p53 or anti-caspases (human) antibodies. The wells were rinsed twice in PBS, incubated for 20 min with a 1:1000 dilution of goat anti alkaline phosphate conjugated IgG antibody (Bangalore Genei, India) for 45 min, rinsed three times in PBS and then incubated for 40 min in p-nitro phenyl phosphate substrate (Sigma). The reaction was stopped with 1.5 M NaOH solution and optical density reading was taken at 405 nm in ELISA reader (ECIL, Hyderabad).

**Results**

**Morphological Examination of Dual Culture**

The sporidia of *T. indica* after germination produced white mycelia, which covered around 25% of the total visible surface of calli within 3 days of growth and all the surface of calli of wheat, barley and rice after 7 days of growth. There were no remarkable differences in growth patterns of calli in terms of wet weight and volume (Fig. 1). All the calli started browning after 15 days of growth. The mycelia developed a brownish creamy appearance and calli became very soft and blackish brown in colour after 30 days of the culture. Both control and dual cultured calli of host and non-host showed plasmolysis with obvious change in its extent among them.

**Immuno-histochemical Localization of Fungal Growth and Development**

In order to study the localization of mycelial growth and chlamydospore like structure in dual cultured calli, immuno-histochemical staining was done in fungal colonized calli of HD 29 (resistant) and WH 542 (susceptible) (Fig. 2). The dark stained
Fig. 1—Establishment of dual culture of resistant (HD 29) and susceptible (WH 542) wheat calli after inoculation of T. indica and its morphological studies. The dual culture of wheat-T. indica, with appropriate control established by adding 200 sporidia on each callus, and maintained in maintenance medium with 20 ppm fungicide (Captan 75 wp) and observed for mycelial growth at different time intervals, a 3 days after inoculation; b 14 days after inoculation; & c 30 days after inoculation. Upper lane-Control; Lower lane- dual cultured calli.

mycelial aggregates were detected within a semi-circular cavity formed by fungal invasion in callus tissue of resistant line. The peripheral cells of the cavity of callus tissue formed a defence barrier by depositing some substances released in resistance response (Fig. 2c). However, no such cavity was formed by fungal colonization in susceptible line. The prominent intensity of gold labelling using antimycelial antibodies throughout the fungal colonized callus tissues and random distribution of mycelial aggregates was observed on disruption of the callus tissues of susceptible line (Fig. 2b). The intensity of antigen as revealed by anti-teliospore antibodies and gold-IgG complex was very faint as only few dark coloured stained particles were observed in resistant line as compared with susceptible line (Figs 2d & e). The susceptible line also showed plasmolysis of cells by fungal invasion.

**Immuno-detection of Cell Death Proteins**

The DIBA results revealed that the expression of p53 started after 14 days of co-culture, increased up to 21 days and then plateau at 30 days of culture (Fig. 3A). The results of periodic changes in p53 levels by microtitre ELISA were almost similar in both susceptible and resistant lines at each time interval of co-culture (Results not shown).

The Caspase-1 was neither activated in resistant line nor in susceptible line during the growth of T. indica at each time interval of observation in dual culture (results not shown). Caspase-3 expressed at 3 days of co-culture, increased steadily up to 21 days and became static at 30 days after the establishment of dual culture. However, there was a remarkable difference in expression levels of Caspase-3 by
Assessment of Genotypic Variation for the Induction of Cell Death Proteins in Dual Culture System

In order to assess the cell death proteins as potential biomarker for screening of host and non-host resistance/susceptibility to KB, cultivars of wheat (8), rice (1) and barley (1) were screened for the differential expression of p53 Caspase-1 and Caspase-3 using microtitre ELISA at 30 DAI.

The ELISA reactivity of cell death proteins showed no expression of Caspase-1 in the dual culture calli of all the cultivars of wheat, barley and rice. However, expression study of Caspase-3 in all the 10 cultivars of host and non-host with goat antirabbit Caspase-3 antibody showed a remarkable difference in the ELISA reactivities (Fig. 4). The results indicated that the expression level of Caspase-3 were highest in the susceptible lines followed by moderately resistant, resistant and non-host cultivars to KB in a decreasing order. After 30 DAI, the levels of Caspase-3 in terms of ELISA reactivity were found to be OD405=0.60 for resistant lines, OD405=0.70 for moderately resistant and OD405=0.90 for susceptible lines. However, the expression studies with p53 antibodies revealed no variation in ELISA reactivity (in the range of OD 0.70) indicating a similar expression level of p53 in all the 10 host and non-host cultivars (Results not shown).

Discussion

Selection of wheat germplasm resistant to T. indica is essential for successful management of KB disease. Screening of wheat varieties, although they differ in their degree of susceptibility to KB pathogen under field conditions, is difficult due to experimental, agricultural and natural constraints. In the present
study, under laboratory conditions, calli derived from host and non-host have been co-inoculated with T. indica to see its response against callus cells of different cultivars showing resistant, moderately resistant and susceptible reaction.

The mycelial growth constantly increased, mycelial mass protruded outward on the calli of both host (susceptible and resistant line) and non-host with no morphogenetic difference. The necrosis pattern was different in host and non-host calli, with more prominent necrosis in susceptible ones (WH 542, HD 2328 and Raj 1555) than moderately resistant (UP 2338, PBW 343) and resistant lines (HD 29, HD 30). The results of immuno-histochemical studies revealed that the mycelia were restricted to a semicircular groove in resistant line HD 29, which represents a common resistance mechanism of plants to the invading fungus. No such groove was observed in susceptible line WH 542. The mycelia, interwoven with host tissue in intercellular spaces, caused plasmolysis (necrosis) of host cells. In dual culture system, the fungus proliferates in the space created by normal disintegration of callus tissues and shrinkage of callus cells provides space for subsequent fungal development like chlamydospore formation. The anti-teliospore antibodies revealed that the chlamydospores share some antigenic homologies with intact teliospore and the formation of chlamydospore like structures were more in susceptible line, WH 542 than the resistant line, HD 29 and the extent of necrosis was more prominent in susceptible line of wheat.

Studies on fungal colonized tissues have further suggested that KB pathogen may cause differential level of PCD in host and non-host plants. Hence the role of PCD was elucidated in pathogenesis and expression of genes linked with it was used as index of susceptibility. In our laboratory, expression of p53 and caspases, crucial cell death proteins, and correlation between their expression and extent of fungal colonization were monitored in dual culture system following pathogen infection in both susceptible and resistant genotypes of wheat. The p53 levels remained almost similar in resistant and susceptible line at each point of observation and there was no remarkable difference in expression among the host and non-hosts. Therefore, p53 cannot be used as a biomarker for defining the host susceptibility/resistance to KB. However, p53 level increased with disease progression and fungal colonization up to 21 DAI and the levels get plateau thereafter. It was observed by microscopic examination that the host cells in callus tissues were strongly plasmolysed when observed at 21 days after inoculation in susceptible line as compared with resistant line. This further suggested that fungal colonization in callus tissues might elaborate certain elicitors/toxic metabolites, which may kill callus cells via p53 dependent apoptotic cell death pathways.

The expression studies of caspases revealed that, there was no expression of caspase-1 in post inoculation period with T. indica. Even in non-host there was no expression of caspase-1, which is expressed, only in inflammatory response of the apoptotic cell death mechanism in animals. The lack of expression of caspase-1 in dual cultured calli of both hosts and non-hosts clearly indicate that plants probably do not have the inflammatory response following fungal colonization, as it is quite obvious phenomenon in animal system. However, caspase-3 expression was observed at 7 days after inoculation and there were differences in its expression levels among host and non-host lines. The level of caspase-3 increased steadily at 14 and 21 DAI and a remarkable difference in the temporal expression of caspase-3 in between resistant (HD 29) and susceptible (WH 542) lines was observed. The maximum expression of caspase-3 was found at 30 days in both resistant and susceptible lines. At this stage the expression level of caspase-3 was much higher in susceptible line than in resistant line.

The expression levels of caspase-3 in hosts and non-hosts, which followed a decreasing trend, correlated well with degree of susceptibility as higher levels were observed in most of the susceptible lines WH 542, HD 2329, Raj 1555 followed by moderate susceptible and resistant lines PBW 343, UP 2338, HD 29, HD 30, PDW 215 as well as non-host barley (HBL 371) and rice (4036). Hence, the differential expression on of caspase-3 may be used as a potential biochemical marker for identification of host and non-host resistance to KB (T. indica) both in field and in vitro conditions.

An increase in expression level of caspase-3 in susceptible line (WH542) may be due to lack of resistance response in the form of antioxidant enzymes or probably proteinase inhibitor. In plant and other organisms, protease like caspase (Cysteine protease) activity can be regulated at different levels: by post-translational processing and by specific protease inhibitor proteins (Solomon et al, 1999). Plants possess a large arsenal of these protease
inhibitor proteins, which determine the different degree of immunity to Karnal bunt.

The present study clearly describes the prospects of using plant tissue culture technology as a potential tool for understanding the molecular mechanisms of disease progression, fungal pathogenesis and PCD using dual culture system. The dual culture system can also be employed as in vitro screening aid to delineate the KB resistance under defined laboratory conditions.

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References


