

Full Length Research Paper

Induced gene expression in wheat seedlings treated with a crude extract of *Agapanthus africanus* L. prior to leaf rust infection

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It was previously shown that a crude extract of *Agapanthus africanus* L. (Hoffman), applied as a foliar spray to wheat (*Triticum aestivum* L.) seedlings, significantly increased the *in vitro* activities of three pathogenesis related (PR) proteins (β -1,3-glucanase, chitinase and peroxidase). This was the case in both susceptible and resistant wheat lines whether the plants were uninfected or infected with leaf rust (*Puccinia triticina*). The aim of this study was to determine the influence of the *A. africanus* extract on both the intercellular PR-protein profile and PR gene expression in leaf rust infected wheat lines. Pre-treatment of infected resistant and susceptible wheat with the extract led to increased β -1,3-glucanase levels that were higher as compared to the untreated controls. Similarly, treatment with the extract led to greater expression of both the PR3 and PR9 genes in infected resistant and susceptible seedlings as compared to the controls. This is also applied to a retrotransposon protein encoding gene whose expression was strongly induced following extract treatment. The induced expression of all these defence-related genes suggests that the crude *A. africanus* extract has the ability to prime the resistance response of wheat prior to leaf rust infection.

Key words: Wheat leaf rust, induced resistance, priming, gene expression, immunoblotting, crude *Agapanthus africanus* extract.

INTRODUCTION

The genetic and biochemical interaction between a plant and a pathogen is very complex with resistance being mediated on two levels (Jones and Dangl, 2006). For the first, both exogenous and endogenous elicitors in the form of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) respectively, activate PAMP triggered immunity (PTI) in the plant. This response is mediated through different PAMP recognition receptors (PRRs) that initiate one of the most important and effective plant defence mechanisms against various pathogens, namely the activation of

defence-related (PR) genes that encode defence proteins known as pathogenesis-related (PR) proteins (Jung et al., 1993). In turn, these enzymes regulate the synthesis of chemical products involved in the defence mechanism (Gang et al., 1999; Garcia-Garrido and Ocampo, 2002, Ferreira et al., 2007), allowing the plants to survive the infection.

Successful pathogens overcome this basal defence by secreting effector proteins into the plant cell where it suppresses the defence response (Jones and Dangl, 2006). In time, plants have gained the ability to recognize

these effectors through disease resistance (R) proteins, thereby exhibiting effector triggered immunity (ETI). This genetic interaction was first described by Flor (1971) who concluded that if both the *R* and *avr* genes are present, the interaction is incompatible and the plant will survive. Effector triggered immunity is especially effective against biotrophic pathogens (Lukasik and Takken, 2009).

A key component of ETI is the hypersensitive response (HR) in the form of localised cell death (Mur et al., 2008). Systemic acquired resistance (SAR), on the other hand, is expressed in distal parts of the plant away from the primary infection site, where it contributes to long-lasting and broad-spectrum resistance to pathogens that would otherwise cause disease (Ryals et al., 1994; Sticher et al., 1997; Somssich, 2003; Durrant and Dong, 2004). Systemic acquired resistance and the signal networks in the HR are summarized by Gozzo (2003).

An important feature of SAR is the expression of the *PR* genes (Kessman et al., 1994; Datta and Muthukrishnan, 1999). These encoded PR-proteins accumulate in the apoplast and the vacuole and include β -1,3-glucanase, chitinase and peroxidase (Van Loon, 1976; Van Loon and Van Strien, 1999). Some of these PR proteins show *in vitro* antimicrobial activity (Niderman et al., 1995; Morrissey and Osbourn, 1999). β -1,3-Glucanase and chitinase degrade the cell walls of fungi and prevent infection of plant cells in this manner. These PR-proteins accumulate in large amounts at the primary infection site, but also in tissues showing SAR (Stintzi et al., 1993). The role of PR-proteins is essential as it has been shown in mutant studies where the mutant plants had little or no resistance (Neuhaus et al., 1992).

The growing concern about negative environmental effects of fungicides and the appearance of fungicide-resistant pathogen strains is a motivating factor to develop alternative protection methods to protect crops. It is known that different treatments can boost the basal defence response, allowing for a stronger and faster activation of the defence during any subsequent pathogen attack (Conrath et al., 2006; Frost et al., 2008). This defence augmentation is known as priming and includes SAR (Jung et al., 2009) that is associated with salicylic acid (SA) accumulation (Loake and Grant, 2007) and induced systemic resistance (ISR). Induced systemic resistance is mediated by the biotic agents such as plant growth-promoting rhizobacteria (Van Wees et al., 2008). Induced systemic resistance (ISR), can be distinguished from SAR because it mainly functions independently of SA but involves jasmonic acid and ethylene as signaling molecules (Pieterse et al., 2001; Kessler et al., 2006; Ton et al., 2007).

The concept of induced resistance has been expanded to include induction by a wide range of biotic and abiotic agents, including several synthetic and natural plant activators (Garcia-Brugger et al., 2006; Herman et al., 2008). When applied to plants, SA can prime the basal defence response of plants (Mur et al., 2009). On the

other hand, the synthetic plant activator BTH (acibenzolar-S-methyl) also increases resistance in wheat against powdery mildew (*Blumeria graminis*), leaf rust (*Puccinia triticina*) and leaf spot (*Septoria* spp.) (Görlach et al., 1996). While not effective against all pathogens, the BTH induced defence response was stronger than that triggered by SA or jasmonic acid (JA) (Pasquer et al., 2005). When these products are applied before infection, immunity can be obtained against a broad range of attackers (Ahmad et al., 2010). These induced defence responses are often a combination of priming and direct defence activation (van Hulst et al., 2006).

The identification of new plant activators is thus of great importance for the agricultural sector. During a previous study (Cawood et al., 2010), foliar application of a crude *Agapanthus africanus* extract to wheat led to increased PR protein activity and a reduction in disease severity during subsequent *P. triticina* infection. The aim of the current study was to determine whether the induced defence response was due to either a priming effect or direct defence response activation.

MATERIALS AND METHODS

Seeds of two near-isogenic wheat (*Triticum aestivum* L.) lines, either resistant (RL6052 = Thatcher / *Lr15*, accession I-15, BV2005) or susceptible (Thatcher, accession I-49, BV2005) to *P. triticina*, were obtained from the germplasm collection of the Department of Plant Sciences, University of the Free State. Fresh above soil tissue of *A. africanus* was collected in Bloemfontein (29°07' S; 26°11' N), South Africa, during the flowering season in November 2009 and dried at 30°C.

Crude extract preparation as well as cultivation, treatment and infection of wheat seedlings

The preparation of the *A. africanus* crude extract, cultivation of wheat seedlings and their subsequent infection with *P. triticina* were performed as described by Cawood et al. (2010). The *A. africanus* extract was suspended in 30 ml distilled water (1 g L⁻¹) and applied as a foliar spray to 17-day-old (3-leaf stage) wheat plants 48 h prior to leaf rust infection. Control plants were sprayed with water.

Extraction of apoplastic fluid from wheat leaves

To obtain a representative sample for each time interval, whole seedlings were randomly harvested from different pots at 0, 24, 48, 72, 96 and 144 h post infection (hpi) and used to collect apoplastic or intercellular wash fluid (IWF) (Cawood et al., 2010). The IWF protein concentration was determined according to Bradford (1976) using Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA) and gamma globulin (Sigma, St Louis, MO, USA) as standard.

Immunoblotting

A total of 30 μ g IWF protein was resolved on 12% (w/v) sodium

Table 1. Nucleotide sequences of primers used in this study.

Gene name	Nucleotide sequence	Annealing temperature (°C)
18S F	5'- CAACTTTTCGATGGTAGGATAG - 3'	50
18S R	5'- CTCGTTAAGGGATTAGATTG - 3'	50
β -1,3-glucanase (PR2) F	5'- TCCACGGCGGTCAAGATGA - 3'	61
β -1,3-glucanase (PR2) R	5'- GGTTCTCGTTGAACATGGC - 3'	61
Chitinase (PR3) F	5'- AAGACGGCGTTGTGGTTCTG - 3'	61
Chitinase (PR3) R	5'- GTAGCGCTTGAGAACCCGAT - 3'	61
Peroxidase (PR9) F	5'- ATCAGACCGTCTCCTGCG - 3'	55.4
Peroxidase (PR9) R	5'- GCAGCTGAGCCTGATCTG - 3'	55.4

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels according to Laemmli (1970). Two identical gels were prepared; one was stained with Coomassie Blue (Zehr et al., 1989) while the other was used for immunoblotting. The separated polypeptides were electroblotted to nitrocellulose membranes (Hybond-C, extra membrane, Amersham Biosciences) using a transfer buffer containing 25 mM Tris-HCl pH 8.3, 192 mM glycine and 20% (v/v) methanol. The membranes were quenched with 8% (w/v) fat-free milk powder in Tris-buffered saline (TBS; 10 mM Tris-HCl pH 8.3, 1.5 M NaCl) for 1 h at room temperature. Subsequently, the membranes were probed for 2 h with a 1:9000 dilution of rabbit anti-wheat β -1,3-glucanase primary antibody in TBS containing 4% (w/v) fat-free milk powder. Following extensive washing with TBST [TBS + 0.1% (v/v) Tween-20], the membranes were incubated for 1 h at room temperature in a 1:9500 dilution of the goat-anti-rabbit secondary antibody (IgG) in TBS. After sequential washing with TBST containing 0.05% (w/v) SDS, TBST and TBS, the antigenic proteins were visualized using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) as a colour enhancer (Blake et al., 1984).

Total RNA extraction

Total RNA was extracted from approximately 0.1 g ground leaf tissue harvested at 0, 24, 48, 72, 96 and 144 hpi using TRIzol Reagent (Invitrogen) according to the manufacturer's specifications. The RNA was finally dissolved in 100 μ l 0.1% (v/v) DMPC treated water. Residual DNA was eliminated by DNase1 treatment according to the manufacturer's specifications (Fermentas). The quality and quantity of the extracted RNA was confirmed spectrophotometrically as described by Sambrook et al. (1989).

Expression analysis of PR genes

The RobusT II RT-PCR kit (Finnzymes) was used to determine the expression pattern of selected PR genes using gene specific primers (Table 1). Each 10 μ l reaction contained 10 ng total RNA, 25 pmol of each primer, 0.2 mM dNTP's, 1.5 mM MgCl₂, 1 x final concentration of the supplied buffer and 0.4 μ l of the RobusT II enzyme mix. The amplification regime was as follows: one cycle at 48°C for 30 min and 94°C for 2 min, 30 cycles at 94°C for 30 s, specific annealing temperature (Table 1) for 30 s and 72°C for 2 min followed by one cycle at 72°C for 5 min. The 18S rRNA gene was used as reference. The amplified products were finally separated on a 1% (w/v) agarose gel (Sambrook et al., 1989).

Sequencing of amplified PR gene fragments

All amplified DNA fragments were sequenced to confirm their identity

Amplified cDNA fragments were cut from the agarose gel, purified using the Favorprep Gel/PCR Purification Kit (Favorgen Biotech) and re-amplified with the KAPA Taq DNA Polymerase Kit (KAPA Biosystems). The DNA fragments were sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Sequenced fragments were ethanol-precipitated and separated on an ABI377 Sequencer (PE Biosystems). The resulting sequences were used to do a BLAST analysis (v 2.2.13) Altschul et al., 1990) with entries available on GenBank.

RESULTS

Polypeptide accumulation in IWF

Infection of the resistant Thatcher/Lr15 line with *P. triticina* led to the accumulation of a 31 kDa polypeptide in the IWF starting at 24 hpi (Figure 1B). No such accumulation was seen in the infected susceptible control plants (Figure 1A). Pre-treatment of wheat seedlings with the *A. africanus* extract 48 h prior to infection resulted not only in the stronger accumulation of the 31 kD polypeptide in the resistant line (Figure 1D), but also in the susceptible line starting at 48 hpi (Figure 1C).

Immunoblotting of the IWF from infected susceptible and resistant wheat previously treated with the *A. africanus* extract revealed that the β -1,3-glucanase antibodies cross-reacted with a polypeptide of ca. 31 kDa (Figure 2). In both cases, an increase in polypeptide levels from 24 hpi onwards was seen. This corresponds with the induced 31 kD protein that was seen on the SDS-PAGE gels. It is thus clear that treatment with the *A. africanus* extract stimulated the production of the β -1,3-glucanase protein in wheat.

Expression of PR-genes

The expression patterns of different PR genes following extract treatment and pathogen infection are presented in Figures 3 and 4. The constitutive expression of the 18S rRNA reference gene in all samples confirmed that equal quantities of RNA were used for all RT-PCR reactions. Constitutive expression of the PR2 gene occurred in both

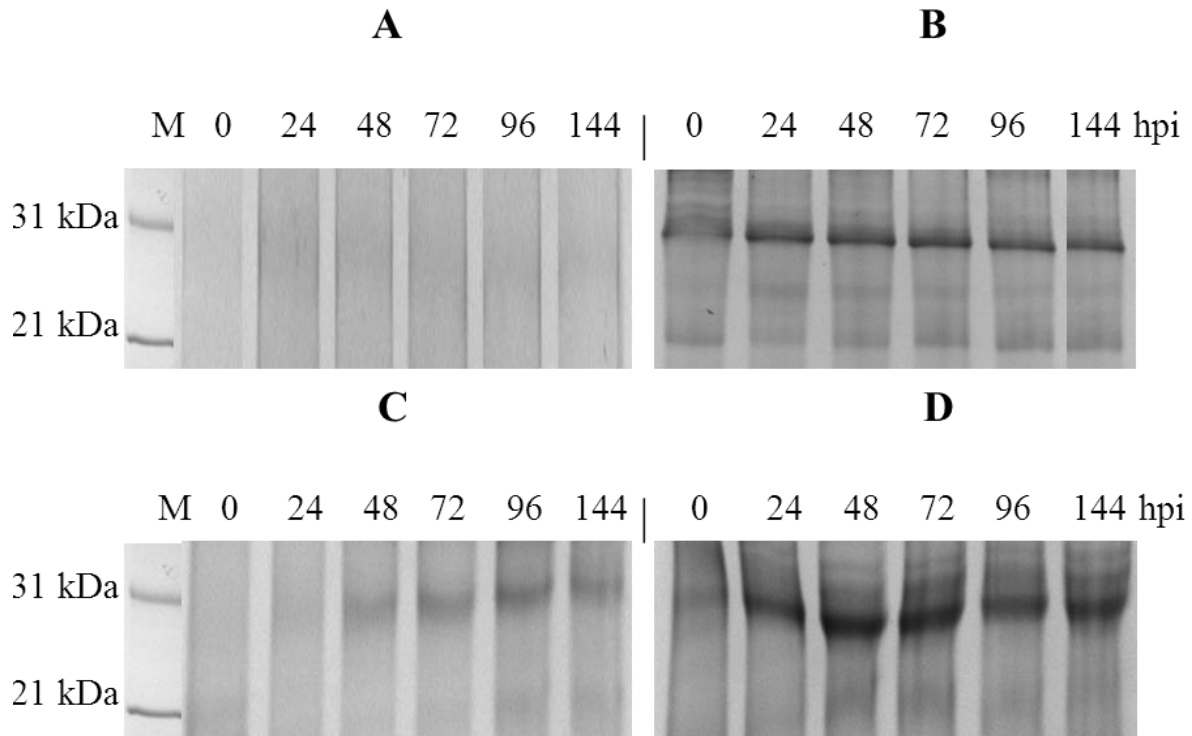


Figure 1. Polypeptide profiles of IWF from leaves of inoculated (A) susceptible Thatcher, (B) resistant Thatcher/*Lr15*, (C) susceptible Thatcher treated with *A. africanus* extract 48 h prior to infection and (D) resistant Thatcher/*Lr15* treated with *A. africanus* extract 48 h prior to infection. IWF was collected from leaves sampled at different time intervals after infection with *P. triticina*. Each lane contained 30 μ g protein. M, Molecular mass marker; hpi = hours post infection.

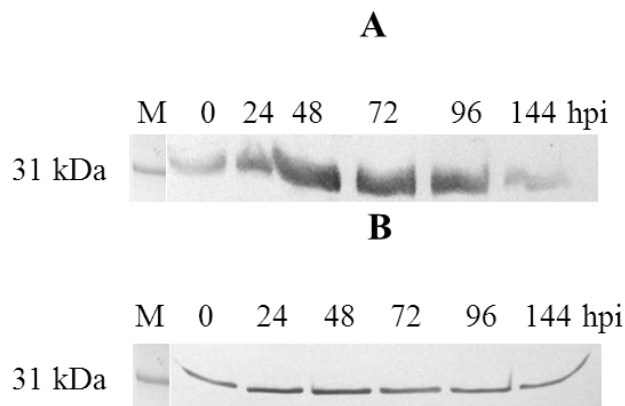


Figure 2. Western blot analysis of β -1,3-glucanase protein of IWF from leaves of inoculated (A) susceptible Thatcher treated with *A. africanus* extract 48 h prior to infection and (B) resistant Thatcher / *Lr15* treated with *A. africanus* extract 48 h prior to infection. IWF was collected from leaves sampled at different time intervals after infection with *P. triticina*. M = Molecular mass marker; hpi = hours post infection.

infected susceptible and resistant control and *A. africanus* treated plants (Figures 3 and 4). The *PR2* gene

appeared to be strongly expressed in the infected susceptible plants treated with *A. africanus* extract as compared to the control plants (Figure 4) with no real visible differences between the treated and control resistant plants inoculated with the pathogen (Figure 3).

Expression of the *PR3* (chitinase) and *PR9* (peroxidase) genes was induced at 24 hpi in both the resistant and susceptible control plants after infection with *P. triticina* (Figures 3 and 4). Treatment with the *A. africanus* extract caused visibly stronger expression of *PR3* and *PR9* gene expression in the infected resistant plants, as well as *PR9* but not *PR3* gene expression in the infected susceptible plants. A strong secondary increase in gene expression for *PR3* was also evident in the extract treated infected resistant plants at 144 hpi as compared to the control. Treatment with the *A. africanus* extract therefore does influence the differential expression of the *PR* genes.

During the analysis of *PR9* expression, a second cDNA fragment approximately 350 bp in size was repeatedly co-amplified with the 750 bp *PR9* fragment. When sequenced, this 350 bp fragment showed 97% identity to *Oryza sativa* clone Oss-289-384-H3 retrotransposon protein mRNA (GenBank accession no. EF576506.1; $1e^{-96}$). Expression of this gene was strongly induced in the infected susceptible wheat over a period of 144 h, but

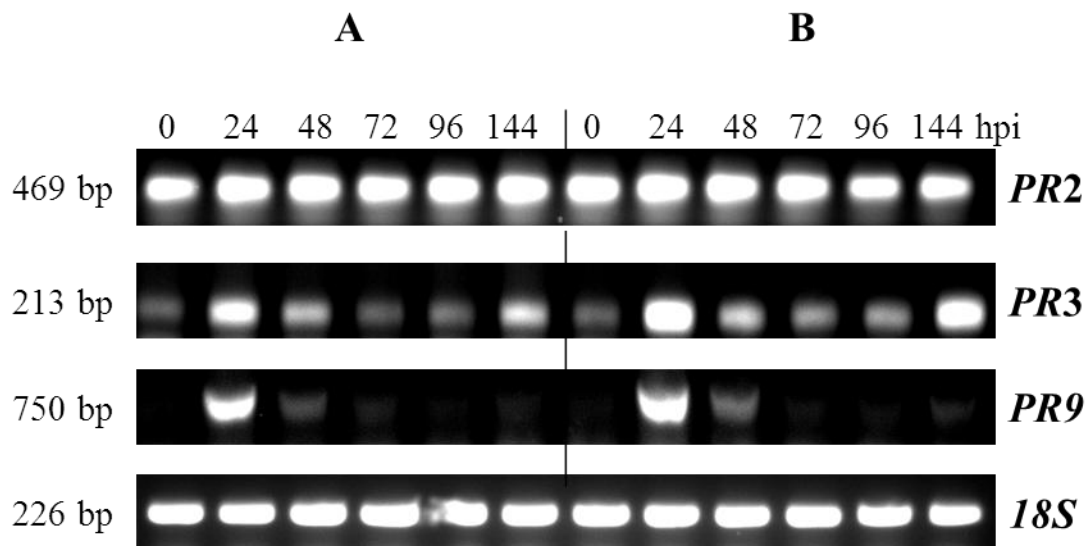


Figure 3. Expression analysis of selected *PR* genes in *P. triticina* infected resistant Thatcher/*Lr15* treated with (A) water and (B) *A. africanus* extract 48 h prior to infection. Time intervals and sizes of the amplified gene fragments are as indicated. Hpi = hours post infection.

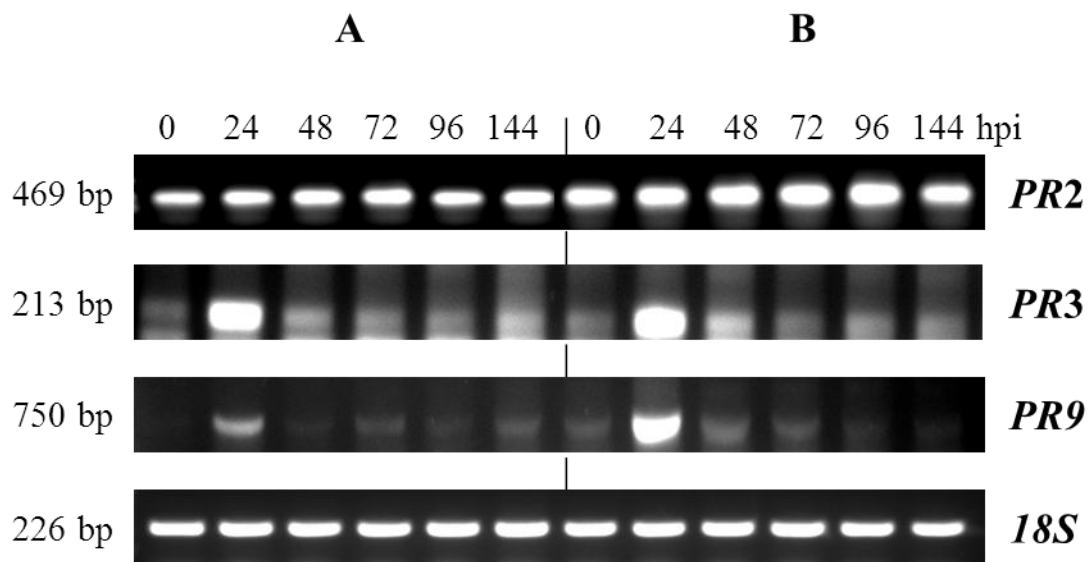


Figure 4. Expression analysis of selected *PR* genes in *P. triticina* infected susceptible Thatcher treated with (A) water and (B) *A. africanus* extract 48 h prior to infection. Time intervals and sizes of the amplified gene fragments are indicated. Hpi = hours post infection.

only to a lesser extent in the infected resistant plants since the basal expression level appeared to be higher in the resistant line (Figure 5). In both *A. africanus* extract treated lines, the expression levels at 0 h was much higher as compared to the controls, indicating that foliar application of the *A. africanus* extract 48 h prior to infection induced the expression of this gene in both wheat lines to its maximum level even before pathogen

infection.

DISCUSSION

Plants naturally express variable levels of resistance against different groups of pathogens. This kind of primary defence response is known as basal resistance

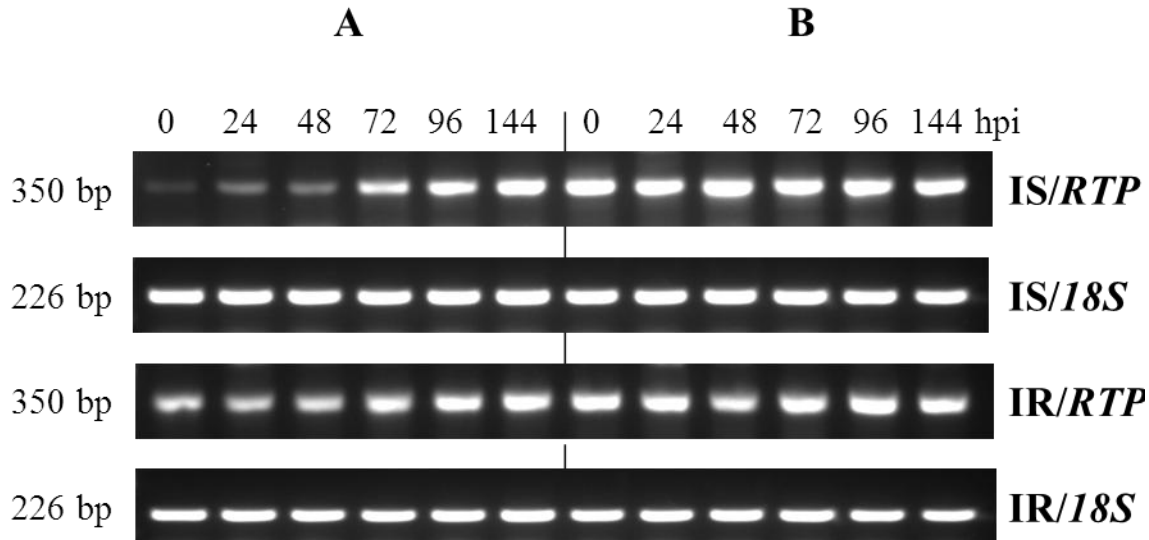


Figure 5. Expression analysis of a wheat retrotransposon protein (*RTP*) encoding gene orthologue in *P. triticina* infected wheat treated 48 h prior to infection with (A) water and (B) *A. africanus* extract. IS indicates infected susceptible Thatcher and IR infected resistant Thatcher/*Lr15* wheat. Hpi = hours post infection.

that is controlled by several genes. It is obtained by cooperation of multiple molecular and cellular defence responses and involvement of various signaling pathways (Jones and Dangl, 2006; Hamiduzzaman et al., 2005). It is considered too weak to be fully exploited in the agricultural sector when compared to race-specific resistance provided by a disease resistance gene, since it cannot completely stop colonization by pathogens.

The mechanism of action of a crude *A. africanus* extract to induce a defence response in wheat was investigated during this study. Analysis of the apoplastic fluid, using SDS-PAGE, indicated the accumulation of a 31 kD polypeptide in the IR plants that was produced at a much higher level in the IR plants previously treated with the extract (Figure 1). Western blot analysis (Figure 2) confirmed the identity of this fragment as being β -1,3 glucanase (Stintzi et al., 1993; Münch-Garthoff et al., 1997). Priming of the defence response by the extract was clearly evident in the IS plants where no β -1,3 glucanase was present in the non-treated control plants, but it accumulated to high levels in the IS plants previously treated with the extract. This confirmed that the previously observed increase of *in vitro* β -1,3 glucanase activity was the result of *de novo* protein synthesis and not merely the activation of existing enzymes (Cawood et al., 2010).

Since the apoplast is recognized as the site where many defence related compounds accumulate (Fink et al., 1988; Van der Westhuizen and Pretorius, 1996; Van der Westhuizen et al., 1998), it was postulated that the accumulation of these proteins in the IWF of plants treated with the *A. africanus* extract may be associated with a compound or compounds in the extract that act as

elicitors of the defence response in wheat. In order to verify this postulate, the expression patterns of selected *PR* genes in infected wheat treated with the extract was determined.

Prior treatment of susceptible and resistant wheat with the *A. africanus* extract followed by *P. triticina* infection, led to the stronger expression of *PR2*, *PR3* and *PR9* genes as compared to the controls (Figures 3 and 4). Combined with the protein work, it is clear that treatment with the extract primed the defence response of the wheat, indicated by the stronger defence response which was only evident following *P. triticina* infection.

Since McClintock (1950) first predicted the existence of 'jumping genes' in the maize genome, transposable elements have been identified in almost all plant species that have been investigated (Okamoto and Hirochika, 2001). Transposons are classified into two classes based on their mechanism of transposition namely Class I: Retrotransposons and Class II: DNA transposons (Casacuberta and Santiago, 2003). Retrotransposons can move around in the genome by means of a "copy and paste" mechanism where RNA copies are reverse transcribed to DNA before being inserted back into the genome. In the process they may cause mutations or increase/decrease the amount of DNA in the genome.

It has been shown that stress conditions can induce the expression and subsequent transposition of retrotransposons through salicylic acid and 2,4-D as part of the defence response (Beguiristain et al., 2001). This is most likely due to the possible exchange of promoter regions between plant defence genes and the retrotransposons (Takeda et al., 1999). In the current study, it was shown that *P. triticina* infection also induced the expression of

the fortuitously identified retrotransposon protein encoding gene (Figure 5). However, treatment with the *A. africanus* extract did not prime the expression of the gene, but rather directly induced the expression thereof.

In accordance with Benhamou (1996), the current study suggests that the *A. africanus* extract contains active compounds that act as elicitors in an induced systemic defence response towards *P. triticina* in wheat. Extract treatment targeted the wheat defence response on three levels, namely increased defence protein activity (Cawood et al., 2010) as well as primed and directly induced gene expression, potentially making this extract a very valuable tool in the agricultural sector. The active compound(s) from *A. africanus* is currently being isolated and purified by means of NMR-spectroscopy with the aim of identifying it and elucidating its chemical structure.

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