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Chapter 5

Russian wheat aphid (*Diuraphis noxia*) induced protein alterations in wheat

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5.1 Abstract

Several studies were undertaken to identify potential sources of resistance towards the Russian wheat aphid (*Diuraphis* noxia) since its detection in South Africa during 1978. Studies conducted on the intercellular washing fluid of wheat resistant to the Russian wheat aphid ('Tugela *Dn1*'), showed that proteins were induced within six days of infestation. These induced proteins were visible as five bands using SDS-PAGE analysis. Two proteins disappeared from the protein profile of 'Tugela *Dn1*' when the Russian wheat aphid was allowed to feed. Analysis of these proteins using two-dimensional gel electrophoresis indicated that these five induced protein bands corresponded to seven proteins. Subsequently, the ≈ 20 kDa band observed with SDS-PAGE analysis, was revealed as three proteins using two-dimensional gel electrophoresis with pI values of 5.0, 5.2 and 5.8. The induced proteins could not be sequenced because of their low concentrations in relation to the other proteins. Overexpression and underexpression of proteins were also visible after Russian wheat aphid infestation. Two of the induced proteins (≈ 36 and 26 kDa) are possibly β-1,3-glucanases. The other unique proteins in this study have not been identified.

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5.2 Introduction

When a plant is exposed to adverse environmental stimuli, certain plant genes are activated that lead to an induction of proteins that partake in the defense response of the plant. The apoplast (intercellular space) plays an important role in the defense strategy of the plant with it being the site where signals originate and also were many defense-related proteins accumulate including the pathogenesis-related (PR) proteins (Bowles, 1990).

The RWA follows an intercellular path towards the phloem with its stylet (Fouché *et al.*, 1984). RWA feeding on resistant wheat, wheatgrass and barley culivars spend considerably less time feeding from the phloem when compared to RWA feeding on the susceptible cultivars. RWA on the resistant cultivars subsequently turned to nonphloem feeding to survive (Kindler *et al.*, 1992; Webster *et al.*, 1993). Van der Westhuizen & Pretorius (1996) found an induction of four groups of proteins in the intercellular fluids of a RWA infested resistant wheat cultivar, 'Tugela *Dn1*'. No induction of proteins was found in the susceptible wheat cultivar ('Tugela') during RWA infestation. Some of the induced proteins were serologically related to PR proteins and the hypothesis was made that these induced proteins are involved in the resistance the RWA encounters when feeding on resistant cultivars. The RWA subsequently encounters these induced proteins when searching for the phloem and, when engaged in nonphloem feeding on resistant cultivars, could possibly ingest these proteins.

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Other studies have also shown the induction of proteins in resistant cultivars during RWA infestation (Porter, 1992; Miller *et al.*, 1994; Rafi *et al.*, 1996; Botha *et al.*, 1998; Van der Westhuizen *et al.*, 1998; Porter & Webster, 2000). Although some of these proteins have been serologically related to proteins like chitinase, β-1,3-glucanase and proteins that possess antifungal activity, many of the induced proteins still need to be identified. More investigations into the exact nature and mechanisms of the induced proteins are necessary to understand their role in the resistance encountered when the RWA feeds on a resistant plant.

In this study, the induction of proteins was examined in RWA infested and uninfested wheat, 'Tugela *Dn1*' ('Tugela*5/SA 1684', a RWA resistant cultivar). These induced proteins were analyzed using SDS-PAGE as well as two-dimensional gel electrophoresis so that these proteins could possibly be identified.

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5.3 Materials and Methods

5.3.1 Plants

Wheat (*Triticum aestivum*) was grown in a greenhouse at a temperature of 25±1°C. RWA was maintained on the 'Palmiet' wheat cultivar, which is RWA susceptible. 'Tugela *Dn1*' was grown to the two leaf growth stage (stage 12; Trottman *et al.*, 1979). At this point the plants were infested with RWA. Aphids were collected with a fine horse-hair paint brush (Aalbersberg *et al.*, 1987) and gently tapped onto the wheat plants to be infested. Approximately five to six aphids were scattered onto each plant. 'Palmiet' plants that were infested with RWA were also placed next to the plants to be infested, so that the RWA could move off the 'Palmiet' plants onto 'Tugela *Dn1*' plants. Control plants were not infested with the RWA.

5.3.2 Intercellular washing fluid (IWF) extraction

IWF was extracted after six days of RWA infestation (Van der Westhuizen & Pretorius, 1996. IWF was extracted according to the method of Rohringer *et al.* (1983). The upper 8 cm of the wheat leaf was used. The cut ends were rinsed twice with distilled water. The leaves (1 g in fresh weight) were then placed with their cut ends first into a glass tube with buffer. The buffer consisted of 50 mM Tris-HCl (pH 7.8) containing 0.5 mM PMSF (phenylmethylsulfonyl fluoride) and 5 mM mercaptoethanol (Van der Westhuizen & Pretorius, 1996). The glass tube was vacuum infiltrated for 30 minutes. The buffer was

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infiltrated by slowly releasing the vacuum. The leaves were removed and quickly blotted dry on paper toweling. The leaves were then centrifuged at 500 *g* for five minutes at 4°C with their ends facing downwards. At the bottom of the centrifuge tube a perforated plastic disk was placed to provide space for the IWF extract. The IWF was recovered after centrifugation and stored at -20°C. The leaves were extracted three times for complete IWF protein collection. IWF was extracted from infested as well as uninfested 'Tugela *Dn1*'.

5.3.3 IWF contamination with cytoplasmic constituents

To establish whether the IWF had been contaminated with intracellular proteins, a malate dehydrogenase (MDH) assay was performed (Cooper, 1977). The assay was carried out at room temperature. Twenty μl of protein sample (corresponding to 5 μg protein) was added to 3.75 mM 1,4-dithiothreitol, 7 mM $MgCl_2$, 0.25 mM NADH, 2.3 mM oxalacetic acid and 80 mM phosphate buffer (pH 7.5) to make up a final volume of 1ml. The reaction was started by adding oxalacetic acid. The oxidation of NADH giving malate was followed spectrophotometrically at a wavelength of 340 nm.

Total protein was extracted from infested 'Tugela *Dn1*' using the method of Van der Westhuizen *et al.* (1998). Leaf tissue was frozen in liquid nitrogen and ground into a fine powder. Proteins were extracted with a 50 mM Tris-HCl buffer (pH 7.8) that contained 2 mM PMSF and 10 mM mercaptoethanol. After centrifugation at 10 000 *g* for 10 min the supernatant was removed and used for the MDH assay.

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MDH activity from the IWF protein and total protein extraction was determined for 'Tugela *Dn1*' infested with the RWA and was replicated three times. MDH activity was expressed as µmol NADH.g^{-1} leaf fresh weight.min^{-1}. MDH contamination of less than 2% indicated little cytoplasmic contamination (Botha *et al.*, 1998).

5.3.4 Protein determination

The protein concentration was determined according to Bradford (1976) using the Bio-Rad protein assay reagent with gamma globulin as the standard.

5.3.5 SDS-PAGE analysis

Proteins were separated by SDS-PAGE (Laemmli, 1970) using a mini gel system containing 15 and 5% acrylamide (acrylamide: bisacrylamide, ratio of 37.5: 1) for the running and stacking gels, respectively (Van der Westhuizen & Pretorius, 1996). Electrophoresis was carried out at a constant voltage of 200 V.

IWF proteins were precipitated with 9 volumes of ice-cold acetone. This was placed at -70°C for 1 hr followed by centrifugation at 15 000 *g* for 20 min. The supernatant was removed and the pellet allowed to dry. The pellet was resuspended in 50 mM Tris (pH 7.8). Twelve µg of protein was loaded into the wells. Gels were then silver stained (Dunn, 1996). Samples loaded were IWF proteins of infested and uninfested 'Tugela *Dn1*'.

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5.3.6 Two-dimensional gel electrophoresis

Isoelectric focusing was performed on the RWA infested and uninfested 'Tugela *Dn1*' IWF samples. The gel solution contained 5.5 g urea, 2.5 ml double distilled water, 1 ml acrylamide/bisacrylamide solution (40% T[1], 5% C_{bis}[2]), 2 ml of a 10% Nonidet P-40 solution, 0.4 ml ampholyte (pH 5-7), 0.1 ml ampholyte (pH 3-10), 7 µl *N, N, N, 'N'*-tetramethyl-ethylenediamine (TEMED) and 10 µl 10% ammonium persulphate. Gels were then cast in glass tubes that had a 1 mm diameter and were 15 cm long. They were allowed to polymerize for 1 hour at room temperature (Oosthuizen *et al.*, 2001).

The gel tubes were then placed in the upper chamber of a Hoefer SE600 gel electrophoresis unit. The anode solution consisted of 10 mM phosphoric acid and the cathode solution of 10 mM histidine. Five µl of sample overlay solution (9 M urea and 2% ampholytes) was added to the top of each gel (Oosthuizen *et al.*, 2001). Gels were then prerun as follows:

i) 200 V for 15 min

ii) 300 V for 30 min

iii) 400 V for 30 min

Protein samples (100 µg) were concentrated according to the method of Wessel & Flugge

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[1] Percentage of the total for acrylamide and bisacrylamide

[2] Concentration of bisacrylamide

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(1984) and resuspended to make a final volume of 20 µl. The protein samples were mixed with 5 µl sample buffer (9.5 M urea, 2% Nonidet P-40, 2% ampholytes and 5% mercaptoethanol). This was then loaded into the pre-focused gels and overlayed with 5 µl sample overlay solution. The IEF gels were then run at 400 V for 16 h and then at 800 V for an additional hour (Oosthuizen *et al.*, 2001).

After IEF, the tube gels were squirted out with dH_2O and equilibrated in equilibration buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 5% mercaptoethanol) for 20 min (Oosthuizen *et al.*, 2001).

A uniform SDS-polyacrylamide separating gel (15% T, 2.7% C_{bis}) was prepared. The IEF gel was sandwiched onto the SDS-PAGE gel with an agarose sealing solution (0.5 M Tris-HCl pH 6.8, 1% SDS, 1% agarose). The SDS-PAGE gel was run at 5 W for 15 min followed by 10 W for 5 h 15 min. A constant temperature of 18°C was maintained during IEF and SDS-PAGE electrophoresis (Oosthuizen *et al.*, 2001). After electrophoresis, gels were silver-stained according to the method of Dunn (1996). Two-dimensional gel electrophoresis of the IWF protein samples of infested and uninfested 'Tugela *Dn1*' were replicated three times to ensure reproducibility of the protein profiles.

5.3.7 Analysis of protein profiles

The IWF sample of the uninfested 'Tugela *Dn1*' and the RWA infested 'Tugela *Dn1*' were replicated three times to ensure consistency of the protein profiles obtained. As the

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number of spots obtained from the two-dimensional gel electrophoresis were few (due to only intercellular proteins being used), the spots were analyzed visually. The number of protein spots per gel was determined and the distinct differences between the two gels were marked.

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5.4 Results

IWF was extracted from RWA uninfested and infested 'Tugela *Dn1*' to investigate the induction of proteins in the apoplast, which is the route that the RWA stylet takes to reach the phloem (Fouché *et al.*, 1984). A MDH assay was done to determine if there was any contamination from intracellular proteins.

MDH contamination. The percentage of MDH contamination is shown in Table 5.1. The percentage MDH contamination when extracting IWF was 0.14%. This is less than 2% which indicates minor cytoplasmic contamination (Botha *et al.*, 1998).

Table 5.1. Malate dehydrogenase assay (MDH) for IWF and total proteins from RWA infested 'Tugela *Dn1*'.

Protein sample	MDH µmol NADH. g^{-1} leaf fresh weight.min^{-1}	Percentage MDH contamination
IWF proteins from RWA infested 'Tugela *Dn1*'	0.28 ± 0.08*	0.14%
Total proteins from RWA infested 'Tugela *Dn1*'	204.60 ± 8.86*	

* The values given are for the average of three replications.

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SDS-PAGE analysis. SDS-PAGE analysis of uninfested and infested 'Tugela *Dn1*' revealed that RWA infestation altered the protein composition of resistant wheat (Figure 5.1). Proteins were induced as well as differentially displayed when comparing the protein profiles. A total of five proteins were unique to the infested 'Tugela *Dn1*' profile (≈ 36, 26, 20 kDa and 2 proteins < 14.2 kDa). Two proteins were only displayed in uninfested 'Tugela *Dn1*' (≈ 29 and 24 kDa). Three proteins were differentially displayed in infested 'Tugela *Dn1*' in comparison to the protein profile obtained for uninfested 'Tugela *Dn1*'. Two proteins were overexpressed (≈ 45 and 22 kDa) and another was underexpressed (≈ 40 kDa) after RWA infestation.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis revealed that there were 48 spots on the uninfested 'Tugela *Dn1*' and 57 spots on the infested 'Tugela *Dn1*' gels (Figure 5.2). As with the SDS-PAGE protein profiles, proteins were induced as well as differentially displayed during RWA infestation (Figure 5.2). Seven protein spots were unique to 'Tugela *Dn1*' during RWA infestation (indicated by diamonds in Figure 6.2B). These unique spots confirmed the same five induced bands obtained with SDS-PAGE analysis (≈ 36 kDa, 26 kDa, three induced proteins all 20 kDa and two induced proteins < 14.2 kDa). As was expected, two-dimensional gel electrophoresis (separation according to protein charge and size) separated proteins of similar size that occurred in the same area on the SDS-PAGE gel.

Proteins whose expression is unique only to uninfested 'Tugela *Dn1*', are indicated by diamonds in Figure 5.2A. As with the SDS-PAGE protein profiles, two proteins were

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found (≈ 29 and 24 kDa). RWA infestation also resulted in the up-regulation of a number of proteins, the most prominent being indicated by an oval (Figure 5.2B).

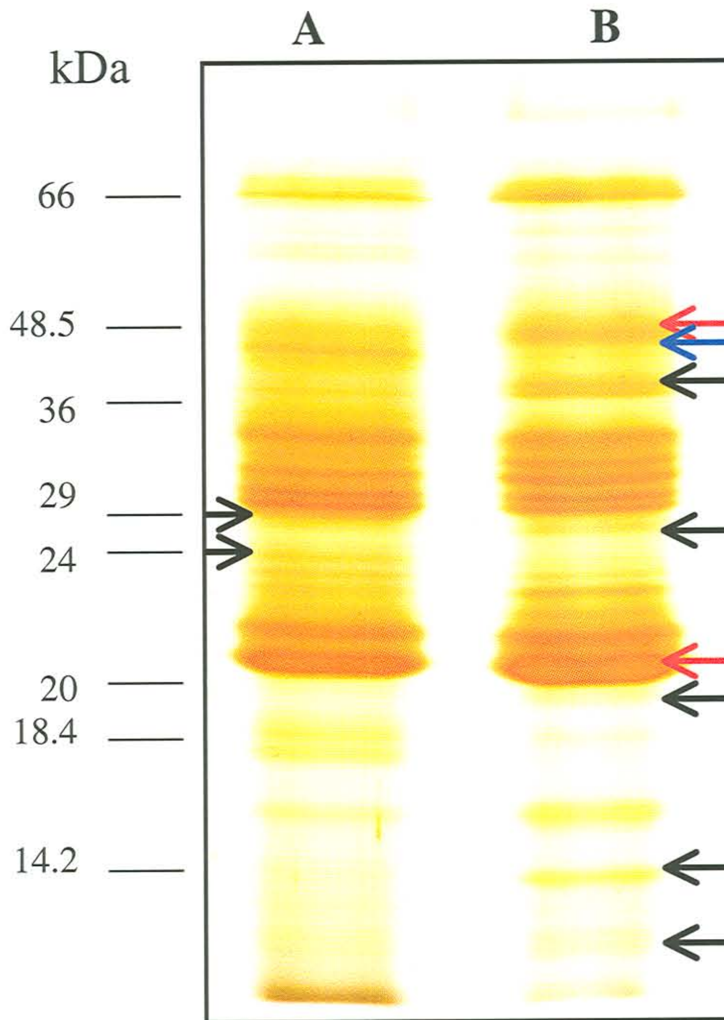

Figure 5.1. SDS-PAGE profiles of intercellular protein from (A) noninfested leaves of 'Tugela *Dn1*' and (B) RWA infested leaves of 'Tugela *Dn1*'. The vertical axis represents molecular masses (kDa). Black arrows indicate unique proteins in each profile. Red arrows indicate proteins that are overexpressed during RWA infestation. Blue arrows indicate proteins that are underexpressed during RWA infestation.

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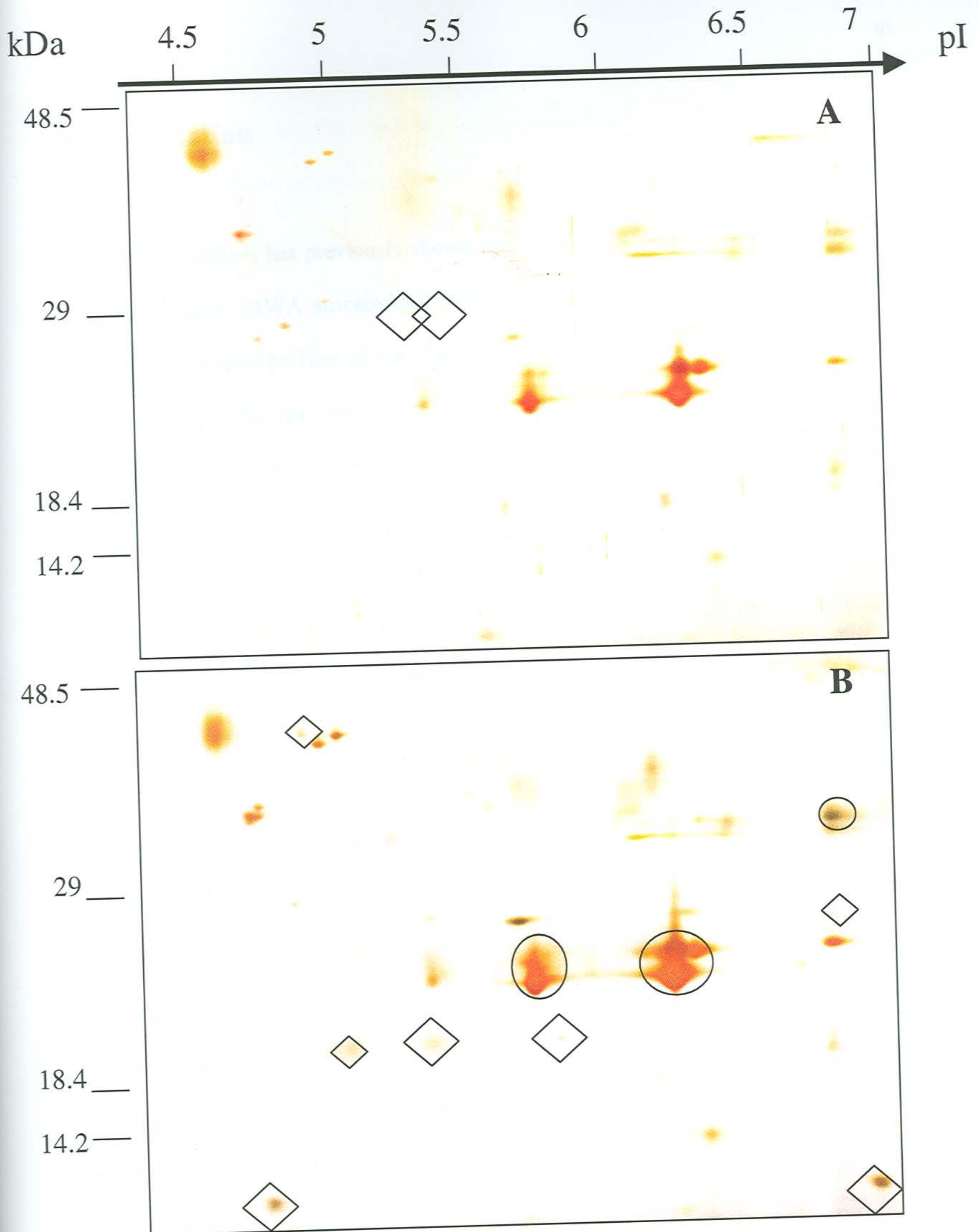

Figure 5.2. Protein profiles after two-dimensional gel electrophoresis of intercellular proteins from (A) noninfested 'Tugela *Dn1*' and (B) RWA infested 'Tugela *Dn1*'. The horizontal axes represents pI's of the isoelectric focusing gradients. The vertical axes represents molecular masses (kDa). Diamonds indicate unique proteins. Ovals indicate proteins that are overexpressed. The protein marker is shown on the left and the pI points to the top of each gel.

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5.5 Discussion

SDS-PAGE analysis has previously shown that protein profiles of RWA uninfested and infested 'Tugela' (RWA susceptible) were similar (Van der Westhuizen & Pretorius, 1996). The protein profiles of the RWA uninfested near-isogenic lines ('Tugela' and 'Tugela *Dn1*') were also similar which indicates the lack of a constitutive resistance factor and also their near-isogenic nature (Van der Westhuizen & Botha, 1993; Van der Westhuizen & Pretorius, 1996).

Induction of proteins was confirmed during RWA infestation of the resistant cultivar ('Tugela *Dn1*'). The five induced proteins seen on the SDS-PAGE profile did not correspond to the induced proteins obtained by Van der Westhuizen & Pretorius (1996). They obtained induced proteins in the following molecular mass ranges: 28-33 (group 1); 22-24 (group 2); 18.5-19.5 (group 3) and 15.5-17 kDa (group 4). Group 1 proteins were serologically related to the β-1,3-glucanase and chitinase PR-proteins and have been implicated in defense against pathogens. The group 2 proteins were serologically related to chitinases and proteins that possess antifungal activity (PR-S proteins). Group 3 proteins appeared to represent a single protein that was overexpressed in RWA resistant wheat ('Tugela *Dn1*'). Group 4 proteins showed two induced bands that could possibly be PR-4 proteins (Van der Westhuizen & Pretorius, 1996).

This study shows an induction of five groups of proteins which corresponds to seven proteins. The sizes of these proteins are different to those found by Van der Westhuizen

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& Pretorius (1996). The first and second induced proteins (≈ 36 and 26 kDa) could possibly be related to the PR-proteins, β-1,3-glucanases and chitinases. These enzymes have sizes that correspond to those of the induced proteins (Fink *et al.*, 1988; Van der Westhuizen *et al.*, 1998). β-1,3-glucanase were found to be induced during RWA infestation of 'Tugela *Dn1*' with the isozymes having pI values ranging from 9.3 to 3.6 (Van der Westhuizen *et al.*, 1998). RWA infestation also resulted in the expression of one chitinase isozyme with a p*I* of 5.5 (Botha *et al.*, 1998). The pI value of the first induced protein in this study was 4.9 and subsequently could be β-1,3-glucanase. The second induced protein has a pI value of 6.8 and is possibly also related to β-1,3-glucanase.

The second group of induced proteins observed by Van der Westhuizen *et al.* (1998), was serologically related to PR-S proteins possessing antifungal activity. There are similarities between a microbial attack on a plant and aphid infestation. β-1,3-glucanases digest cell wall constituents of plants, pathogenic fungi and bacteria and release elicitors that could activate the defense response of the plant. Chitinases hydrolyzes chitin of fungi and insects to protect the plant against attack (Botha *et al.*, 1998). The actual direct role of β-1,3-glucanase and chitinases in plant defense against the RWA is unknown. One hypothesis is that their action releases oligosaccharides which triggers the defence reactions of the plant, similar to the reaction of the plant to hyphal penetration (Dreyer & Campbell, 1987).

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Group 3 proteins described by Van der Westhuizen *et al.* (1998) were not serologically related to any protein and their function is unknown. Occurring closely to this group of proteins was the three induced proteins observed in this study (all ≈ 20 kDa). The last two induced proteins in this study (< 14 kDa) are dissimilar to the group 4 proteins found by Van der Westhuizen *et al.* (1998). Also, the two proteins absent after RWA infestation could not be identified. Protein sequence analysis of induced proteins would help clarify the exact nature of these proteins. Two-dimensional gel electrophoresis has shown that bands obtained from SDS-PAGE analysis represent more than one protein.

Other studies have also indicated that changes in the protein profiles occur during RWA infestation. Miller *et al.* (1994) demonstrated the changes in proteins with two-dimensional gel electrophoresis of total protein from RWA infested 'Morex', a susceptible barley cultivar, and PI 366450, a resistant Afghanistan barley accession (plants were infested for six days). The PI 366450 cultivar showed a shift in the isoelectric point of a 23 kDa complex, with the complex being inhibited in 'Morex'. Porter & Webster (2000) showed an inhibition in a 24 kDa protein complex in a RWA infested susceptible wheat cultivar ('Pavon') that persisted in a RWA infested resistant cultivar (PI 140207). The protein profiles obtained were from total protein extracted from leaves that were infested for four days. Rafi *et al.* (1996) showed using SDS-PAGE analysis, enhanced and reduced expression as well as the appearance of unique proteins from total protein extracts. There was an appearance of a ≈ 53 kDa protein in RWA infested PI 137739 (RWA resistant wheat cultivar) at 11 days. This protein also appeared in the susceptible RWA cultivar ('Stephens') at day six.

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RWA feeding does alter the expression of proteins upon infestation of plants. RWA feeding can cause the overexpression and underexpression of proteins as well as the appearance of novel proteins, as the plant attempts to defend itself against attack. The exact nature and role of these proteins is not known but they do seem to play a part in the defense of the plant against aphid feeding. RWA induced proteins are found in the apoplast (the route the stylet takes) and it has been shown that RWA on resistant plants feed less from the phloem and turn to nonphloem feeding to survive (Kindler *et al.*, 1992; Webster *et al.*, 1993). The induction of proteins (β-1,3-glucanase and chitinase) in the apoplast possibly aids the defense reaction of the plant to invasion (Botha *et al.*, 1998; Van der Westhuizen *et al.*, 1998). Unidentified induced proteins could possibly act as a deterrent to the RWA when it turns to nonphloem feeding on resistant plants. Further research should identify these proteins and their roles tested in artificial diet experiments.

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