ASPECTS OF β-1,3-GLUCANASE EXPRESSION IN WHEAT ASSOCIATED WITH RUSSIAN WHEAT APHID RESISTANCE

BY

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Submitted in fulfilment of the requirements for the degree Magister Scientiae

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November 2003

Preface

The work offered here are results from an original study conducted at the Department of Plant Sciences in the Division Botany at the University of the Free State, Bloemfontein, under the guidance of Prof. AJ van der Westhuizen and Mr. B Visser.

It is known that Russian wheat aphid (RWA) infestation leads to the induction of β-1,3-glucanases in the resistant wheat plants sooner and at higher levels compared to the susceptible plants. The role that β-1,3-glucanase plays during the resistance mechanism are however not understood. This dissertation attempts to shed more light on the possible mechanisms involved.

This research attempts to provide valuable information for other researchers investigating plant-aphid interactions.

I have not previously submitted the dissertation to any other university/faculty. I therefore cede its copyright in favour of the University of the Free State.

Published papers and presentations

Van der Westhuizen AJ, Qian X-M, Wilding M & Botha A-M (2002) Purification and immunocytochemical localisation of a wheat β-1,3-glucanase induced by Russian wheat aphid infestation. **South African Journal of Science 98, March/April 2002**

Wilding M (1998) Regulation of some PR-protein genes after Russian wheat aphid infestation. **Postgraduate symposium, University of the Free State**

Wilding M (1999) Aspects of β-1,3-glucanase expression in wheat after Russian wheat aphid infestation. **Postgraduate symposium, University of the Free State**

Acknowledgements

I would like to thank:

- **Prof. Amie van der Westhuizen and Mr. Botma Visser for their guidance and help**
- **The University of the Free State for the use of their facilities**
- **The National Research Foundation for financial support**
- **All my colleagues and friends for their help**
- **A special thanks to Rouvay, my fiancé, and my family for all their support**

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Abbreviations

BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
BTH	Benzothiadiazole
DEPC	Diethyl pyrocarbonate
DTT	1,4-ditheiothreitol
ER	endoplasmic reticulum
EDTA	(Ethylenediamine)tetraacetic acid
GAR	Goat-anti-rabbit antibody
GM	Genetically modified
IWF	Intercellular washing fluid
kDa	Kilodalton
MDH	Malate dehydrogenase
MOPS	3-(N-morpholino)propanesulfonic acid
NBT	Nitro blue tetrazolium
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PI	Proteinase inhibitors
PMSF	Phenylmethylsulphonylfluoride
PPO	Polyphenol oxidase
PVP	Polyvinylpyrrolidone
RWA	Russian wheat aphid (*Diuraphis noxia*)
RT-PCR	Reverse-transcription polymerase chain reaction
SADC	Southern African Development Community
SDS	Sodium dodecyl sulphate
SSC	Sodium citrate buffer
TBS	Tris buffered saline
TBST	Tris buffered saline Tween
Tris	Tris(hydroxymethyl)aminomethane

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CHAPTER 1

INTRODUCTION

The history of bread revolves around wheat and rye, with wheat being much more prominent. Only wheat, and to a much lesser extent rye, has the protein structures required to retain gas in the leavened dough to produce a light aerated loaf of bread. The Egyptians, who invented bread, based their entire administrative system upon it. The Jews made bread the starting point of their religious and social laws while the Romans conquered the entire world by it and lost the world through it. Bread ruled over the ancient world and even today, bread remains the staple food of many countries (Jackel, 1995).

The origin of the wheat plant is not known with certainty, although a good deal of evidence indicates that cultivated einkorn was developed from a type of wild grass native to the arid lands of Asia Minor (Shellenberger, 1964). The earliest domesticated wheat dates back to approximately 7500-6500 BC and cultivation occurred in the 'Fertile Crescent' including the mountain chains flanking the plains of Mesopotamia and the Syrian Desert, as well as Anatolia and the Balkans. This seems logic because it is the centre of the geographical distribution of the wild progenitors of cultivated wheat of today (Feldman, 1976).

Wheat production dispersed with the spread of agriculture into Europe from Greece. The earliest European agricultural activity can be traced to Yugoslavia and Bulgaria. Emmer and einkorn were the main wheat varieties to be cultivated. From these regions agriculture spread into Hungary, Czechoslovakia, Poland and Germany to the Low Countries, reaching the North Sea coast before 4000 BC (Renfrew, 1973). During the Late Bronze-, Early Iron Age cereal farming based on einkorn, emmer, bread/club and spelta occurred in Sweden, Denmark and Britain (Feldman, 1976).

The Late Bronze Age and the emerging metallurgic industries brought the advent of the plough, which lead to the wide adoption of mixed farming. The First Iron Age provided more durable metals, which were invaluable for agricultural implements. These culminated in increased grain production and an expanding grain trade so that wheat became a very important item in international exchange (Bell, 1987).

Currently wheat is the most important food grain in the world (Dendy and Brockway, 2001). The total wheat production worldwide is estimated at 528 million metric tons. It increased at a rate of 3.3 percent per year between 1949 and 1978. This increase was due to both an expansion of the production area and increased yields per acre. However, since the 1960's,

increased yields were achieved by cultivating improved varieties and by a greatly expanded use of irrigation, pesticides and fertilizers. Currently the aim is to sustain the high rate of growth in wheat production worldwide. The increased production by means of new technologies during this period became known as the "green revolution" (Fowler, 1998).

Maize is the most important grain crop in South Africa and production areas constitute 36 percent of the arable land, with wheat second at 21 percent. A total of 2,317,000 metric tons of wheat was produced during 1995/96 in the Southern African Development Community (SADC) countries. South Africa contributed 91 percent of this. The gross value of this wheat amounted to R1354 million in 1994/95. Its contribution to the value of gross agricultural production has been estimated at 3.59-6.3% over the past decade (Marasas *et al*., 1997).

Wheat is produced mainly in the winter rainfall areas of the Western Cape and the summer rainfall areas of the Free State, Northern Cape, North West and Northern Province. The Free State is the largest wheat producing area, but production is also subjected to considerable annual fluctuations due to the variability in annual rainfall patterns (Wheat Board, 1995/96).

The Central Free State district, the highest wheat production area in South Africa, is also the only region where *Diuraphis noxia* (Mordvilko)(Russian wheat aphid) has an annual occurrence (Wheat Board, 1995/96). RWA is not an endemic species, but was first reported in 1978 near Bethlehem in the Eastern Free State. Since the early 1980's, it had a major impact on the South African wheat industry (Walters *et al.*, 1980).

Initial control consisted of spraying crops with a combination of parathion and one of a series of systemic insecticides. This control measures relied heavily on financial resources and management skills to ensure economically viable RWA management (Du Toit, 1987).

Resistant wheat lines were identified after greenhouse trails at the Small Grain Institute at Bethlehem (Du Toit, 1987, 1988, 1989a). Even though the mechanism of resistance was not understood, backcrossing into wheat lines with more acceptable agronomic characteristics transferred these "resistance genes". In 1992, South Africa released the world's first commercial cultivar with RWA resistance, namely Tugela DN. DN denotes "*Diuraphis noxia* resistance" and more precisely the Dn1 gene or PI 137739. Four other resistance genes further used as resistance resources were PI 262660 (Dn2), PI 294994 (Dn5), Citr2401 and Aus 22498 (Marasas *et al.*, 1997).

Backcrossing the susceptible plants with the resistant donor plant, which yielded near-isogenic lines, produced these resistant cultivars. These resistant cultivars create excellent opportunities to study the resistance mechanisms, because differences in the expression of proteins after infestation by the RWA can only be attributed to the resistance gene. By using the isogenic lines it was shown that some pathogenesis-related (PR) proteins previously associated with resistant mechanisms against pathogens (Archambault *et al.*, 1998) are expressed at much higher levels in RWA infested resistant plants (Van der Westhuizen *et al.*, 1998a). These proteins, amongst others, included β-1,3-glucanases and chitinases.

β-1,3-glucanase and chitinase belong to groups two and three of the PR protein families (Stintzi *et al.*, 1993a). They can participate directly in the defence mechanism against pathogens by means of a hydrolysis function (Mauch *et al.*, 1988a; Sela-Buurlage *et al.*, 1993). Indirectly they are responsible for the release of oligosaccharides which act as elicitors of the defence response (Somssich *et al.*, 1986; Barber *et al.*, 1989; Hughes and Dickerson, 1991).

These PR proteins have been implicated to play a role in the relationship between pathogens and their host plants (Stintzi *et al.*, 1993b). Little is however known of the role of PR proteins in insect-plant interactions. The expression of these proteins after infestation has been shown in previous studies (Van der Westhuizen and Botha, 1993). More information on the expression of β-1,3-glucanase in wheat plants after RWA infestation would therefore be firstly indispensable in understanding the interaction and secondly in the creation of resistant plants through biotechnology.

CHAPTER 2 LITERATURE REVIEW

2.1. THE ORIGIN OF APHIDS

The oldest known fossil aphid, *Triassoaphis cubitus*, is from the Triassic period, the same period as the dinosaurs. However, it is likely Aphidoidea originated from the Archescytinidae in the Carboniferous era, or early Permiam, 280 million years ago. The Aphidinae, which is the largest subfamily of the modern aphids, is not represented in the fossil record until the late Tertiary period. They co-evolved primarily with the Angiosperms and today only very few aphid species live on Gymnosperms, while some attack ferns and mosses (Heie, 1987). It is during this Tertiary period that the modern world, with its characteristic geography, animals and plants as we know them today, came into being ("Cenozoic Era" Microsoft (R) Copyright (c), 1994).

Diuraphis noxia belongs to the super-family Aphidoidae, subfamily Aphidinae, tribe Macrosiphini (Heie, 1992). *D. noxia* have had large outbreaks in Russia before 1900 but was classified as *Aphis cerealis* (Grossheim, 1914). Even though the genus *Diuraphis* is a small genus of about 10 species, it is an economically important genus, causing many crop losses, especially in the Poaceae. Seven species of aphids have been described in Europe and three in North America (Heie, 1992).

2.1.1. Characteristics of *Diuraphis noxia*

The RWA is a relatively small aphid, 1.5 to 1.8 mm in length. Its body is tapered posteriorly with the characteristic double tail feature. The colour is pale yellow green or greyish green and dusted with powdery white wax. Compared to other aphids, the RWA has much shorter antennae, only about one-quarter of the body length (Walters *et al.*, 1980).

2.1.2. Hosts

RWA is a serious pest of wheat and barley. Its ability to utilize a large number of grasses other than cereals increases its likelihood of establishment in new habitats (Kindler *et al.*, 1992). The ability of the RWA to feed on different hosts enables it to over winter on a different host.

Flying aphids, which respond to either olfactory and/or visual cues, colonise plants. After settling, an aphid recognises a potential host by the structure and chemistry of its surface and

internal tissue (Kennedy and Booth, 1951). It has been shown that the RWA is able to select the susceptible plants over the resistant plants from a distance. However, the basis on which the aphid discriminates between resistant and susceptible plants is still unknown (Xinzhi and Quisenberry, 1997).

2.1.3. Distribution

The RWA is indigenous to the cereal-producing regions of the Middle East, Asia Minor and southern Russia (Grossheim, 1914). The distribution has increased dramatically over the last two decades, because of accidental introductions to other cereal-producing areas of the world (Smith *et al.*, 1991). In 1978, *D. noxia* appeared in South Africa (Walters *et al.*, 1980) and in 1980, it was discovered in Mexico (Gilchrist *et al.*, 1984). By 1986, the species had migrated into the South-western USA and spread throughout the western wheat-producing areas of the USA and Canada (Stoetzel, 1987). Genetic analysis of RWAs of different origins showed that the South African RWA shared a common origin with those from France, Mexico and USA and that they may have originated in Turkey (Puterka *et al*., 1993).

After the initial discovery in the Eastern Free State, South Africa in 1978, the aphid quickly spread to the Western Free State and Lesotho and all the wheat producing regions. Isolated infestations were also recorded in Transvaal and Natal (Walters *et al.*, 1980).

2.1.4. Reproductive biology of the RWA

In cold climates, the biology is holocyclic, therefore producing sexual and asexual morphs. In warmer regions, it is probably anholocyclic producing only asexual morphs. In South Africa, the RWA population consists entirely of parthenogenetic females and their life cycle is anholocyclic. The aphids bear their young live and these already have embryos developing inside them. Under ideal conditions, starting from a single aphid, it is possible that a total of 3360 aphids can be produced in as little as six weeks (Pfeiffer, 2001). No sexual reproduction cycle whereby eggs are formed, exists in the South African population (Walters *et al.*, 1980).

The winged females only develop under certain conditions, such as when the host plants are under stress or when the growth stage of the plant is such that it no longer provides a favourable habitat for the aphid. The winged form serves to distribute the aphid to nearby fields or even to other areas (Walters *et al.*, 1980).

2.1.5. Damage caused by the feeding aphids

Yield losses due to aphid infestations have two main causes. Aphids feed directly on the plant sap, thereby reducing the amount of nutrients available for plant growth. A feeding aphid has to ingest large amounts of phloem sap to acquire enough proteins. For example, an adult sycamore aphid requires 2.1 µl sap per day, the contents of 5200 sieve elements. Even a first instar requires 0.8 µl per day, or the contents of 2000 sieve tubes (Dixon, 1973). Therefore, even a small population can have a devastating effect on the availability of nutrients to the plants, which lower the subsequent yield.

With several aphids, an additional cause of yield losses is the transfer of viruses through the saliva that is injected into the plant to dissolve pectate layers of the mid-lamellae. However, in the case of *D. noxia* there is no transfer of viruses, but an unknown toxin is injected into the plant. Fouche *et al.*, (1984) evaluated RWA damage at the ultrastructural level and found that chloroplasts and cellular membranes were destroyed during the feeding process. This total destruction of the chloroplasts is the reason for the yellow leaf streaking. The destruction of the membranes causes disruption of osmoregulatory processes and the occurrence of drought-stress symptoms in the leaves of infested plants. This could be the main reason for the collapse of the leaves under heavy aphid infestation (Riedell, 1989).

Other symptoms include leaf rolling and prostate growth (Walters *et al.*, 1980; Hewitt *et al.*, 1984). The curling of leaves not only reduces the photosynthetic area, but it also provides an optimum environment for aphid reproduction, since contact insecticides together with biological control are incapable of reaching the aphids in the rolled leaves. Another problem resulting from this is that the rolled leaves at the heading stage can prevent spike extrusion, resulting in the obstruction of flowering and decreased seed set (Miller *et al.*, 1994).

All of the above-mentioned factors contribute to plant stunting (Bush *et al.*, 1989), which leads to the most important symptom of RWA infestation, which is lower yield.

2.2. AGRICULTURAL CONTROL OF THE RWA

Insecticides were at first the only way to control the RWA worldwide. One problem associated with the use of poisons is application timing. Chemical control is the most effective at growth stage 31 (Tottman and Makepeace, 1979), when the colonisation of the flag leaf and second leaf can be inhibited and significant yield losses can thus be prevented. The only

problem with this approach is that some damage has already been done to the plant limiting its yield. Reinfestation of the plants after spraying is also possible (Walters *et al.*, 1980).

2.2.1. Alternatives

Any plant in its natural surroundings must be able to survive constant biotic and abiotic stresses induced by its habitat. By natural selection, a plant is able to adapt to its environment and it is this capability, which makes it possible to produce resistant plants (Meins *et al.*, 1992; Stintzi *et al.*, 1993a). To make it possible to survive, wheat plants of Middle East origin must have developed some form of resistance to grow in the region where the RWA is endemic. These resistance mechanisms are currently used with great success to produce resistant wheat where RWA was accidentally introduced.

When the RWA was first detected in South Africa, all the cultivars were susceptible. Sources of resistance were searched for in regions where the aphid is endemic. Of the first successes was the hard red winter wheat cultivar "Amigo", which is resistant to the greenbug, *Schizaphis graminum*, but demonstrated only moderate resistance to *D. noxia* (Butts and Pakendorf, 1984a).

Other potential resistance sources were found in the ancestral wheat species *Triticum monococcum*, *T. timopheevi*, *T. dicoccoides* and *Aegilops squarrosa* (Butts and Pakendorf, 1984b; Du Toit and Van Niekerk, 1985). However, the most successful resistance genes were found in the Soviet Union and Iran (PI 262660, PI 47545, PI 94355, PI 94365, PI 94460 and PI 151918) (Du Toit, 1987; Smith *et al.*, 1991) and Bulgaria (PI 294994) (Du Toit, 1988).

The resistance genes were found to be dominant and are inherited independently (Du Toit, 1989b). However, Zang *et al.*, (1998) showed that the original PI 294994 could be reclassified into four subaccessions, one has one dominant resistance gene, one has two dominant genes, one has one dominant and one recessive resistance genes and two dominant resistance genes in coupling-phase linkages. This led to differences in the class of resistance observed by Budak *et al.*, (1999) in wheat. Differences were also observed when plants containing Dn1, Dn2 or Dn5 were compared. Dn5 plants had much smaller lesion size compared to the other two (Du Toit, 1988). All of these potential resistance genes were tested and the genes, which conferred the best resistance, were backcrossed into cultivars that were adapted to South African conditions. Through this backcrossing technique, resistant cultivars like Tugela DN (Dn1) (PI 137739) and Betta Dn2 (PI 262660) and many other resistant cultivars were bred.

.Phenotypic differences due to the resistance genes are obvious when infested susceptible and resistant plants are compared (2.1.5.). The mechanism behind these differences is, however not yet understood. We need to understand the entire mechanism surrounding the plant-insect interaction to enable us to develop better plants to keep ahead of the evolution of new aphid biotypes that may overcome the resistance we are currently using.

2.3. ENGINEERING GENETIC RESISTANCE IN PLANTS

Gene transfer provides excellent opportunities for enhanced insect resistance in plants (Strauss *et al*., 1991). Many different methods have been employed to produce plants resistant to a variety of pathogens and insects. For instance, transgenic plants that express chitinases, β-glucanases (Broglie *et al*., 1991; Zhu *et al*., 1994) or protease inhibitors (Johnson *et al*., 1989) constitutively at elevated levels have been shown to be resistant to fungi and insects.

Not only genes coming from the same plant species but also genes coming from other organisms have been used to transform plants against pests. A *Bacillus thuringiensis* (Bt) toxin gene from the *Bacillus* bacterium has been used successfully to transform many plant species from forest trees to cotton and corn (Strauss *et al*., 1991; Pannetier *et al*., 1997). This toxin formed by the genetically modified (GM) plant is only toxic to certain species of insects when ingested and is therefore very environmentally friendly (Strauss *et al*., 1991).

Even though many plant transformations have been done and have been shown to be effective against a wide variety of pests and pathogens, there are problems associated with this method of resistance. The biggest problem could be that the insect counter evolves to overcome these resistance traits. Alternatives would be to use multiple genes for resistance thereby making it difficult for a potential pest to overcome the resistance mechanisms (Strauss *et al*., 1991). These multiple genes would have to be several genes coding for entomopathogenic proteins with different modes of action. Combining proteinase inhibitors with the Bt gene would reduce the risk of the appearance of resistant insects, thereby increasing the host spectrum and provide a broader resistance range (Pannetier *et al*., 1997).

While the transfer of PR-genes to plants maybe very useful, it is more effective to transfer multiple genes instead of only one. It was shown that β-1,3-glucanases together with chitinases are responsible for resistance against certain pathogens while separately they were not that effective (Sela-Buurlage *et al*., 1993). Understanding the interaction of these enzymes

and their function in the hypersensitive reaction (HR) in the resistance mechanism would help immensely for future transformations and overcoming possible resistance of the pests.

2.4. PLANT-INSECT INTERACTIONS

Plants and insects have co-existed for at least 100 million years and have evolved a variety of beneficial and deleterious interactions. Pollination benefits both partners with insects cross-fertilising plants in the process of collecting nectar, more often though, herbivorous insects damage plants. Mechanisms of defence against herbivores and microbial pathogens have been evolving for millions of years and are therefore, shared across many plant families (Mitchell-Olds, 1999).

A popular view is that plant populations and their pests are in equilibrium, with the frequency of resistance alleles highest in those populations in which the virulence of the pests is highest or in areas where the pest is common. The frequency of a resistance polymorphism in plant species is closely related to the level of virulence of the plant's primary pest (a specialist herbivore). Evidently there is a co-evolutionary relationship between the plant and the pest species and it is possible that costs play at least some role in maintaining that relationship (Berenbaum and Zanger, 1998).

The cost implicated is the cost of gaining resistance. Studies of resistance to insects in natural plant populations often reveal genetic variation for resistance (Rausher, 1996). Some evolutionary factors can maintain widespread genetic variation for an advantageous trait. Fitness costs of physiological investments in plant defence could maintain this heritable variation. Baldwin (1998) compared fitness of *Nicotiana attenuata* when herbivores were present or absent. In populations with intermediate levels of herbivory, jasmonate-induced plants experienced less insect damage and had higher fitness than uninduced controls. In low herbivory treatments, however, induced plants had lower fitness than their uninduced counterparts. Energetically expensive secondary compounds like nicotine are favourable, however it reduces the plant fitness when herbivory is low. The same is true where high trichome density gives *Arabidopsis* plants higher resistance against herbivore attack, but low trichome density is favoured when herbivores are excluded. The plant just cannot afford to put too many resources into resistance mechanisms while limiting the resources towards reproduction.

As mentioned, plants cannot invest too many resources into resistance mechanisms. It however needs to protect itself against possible attack from pathogens and herbivores. Plants can either invest in constitutive or inducible defences. Constitutive defences are clearly not the answer if you take into consideration the costs of maintaining these defences even in the absence of attack. Inducible defences on the other hand will not be activated until the plant is under attack, thereby limiting the amount of resources directed into defences until it is necessary.

Plant-insect interactions are not so well known as plant-pathogen interactions. However, the little that is known about plant-insect interactions shows high similarities (Van der Westhuizen *et al*., 1998b). By using plant-pathogen interactions as a model, plant-insect interactions can be more readily explained.

According to the resistance model, all plants are naturally resistant to almost all potentially pathogenic organisms (Johal *et al*., 1995). The pathogens have to overcome this non-host or general resistance to colonise the plant and have developed a number of different ways to achieve it. Biotrophic pathogens evade the plant's surveillance system and grow unnoticed inside their hosts (Fig. 2.1). Necrotrophic pathogens produce phytotoxic metabolites that usually interact with specific proteins (receptors or enzymes), which suppress or slow down the plant's defences (Fig. 2.1).

Due to the co-evolution of plants and their pathogens, resistance mechanisms have developed whereby the plant limits the amount of damage caused by the pathogen. Resistance against biotrophs relies on the recognition of the pathogen by the plant due to a signal from the pathogen, which can trigger the HR (Keen, 1990). Resistance against necrotrophs where toxins are involved operates by interfering with the action of the toxin. Therefore, the toxins cannot react with their receptors and the plant defences are not suppressed or slowed.

A quick summary of the plant-pathogen interaction can be illustrated as in figure 2.2. Due to the general resistance in plants, pathogens adapt to overcome this resistance using different mechanisms (biotrophic or necrotrophic). The plants developed resistance mechanisms during the co-evolution in which a specific interaction (gene-for-gene) causes a resistance against that specific pathogen. The most important character of a resistant plant is the plant's ability to recognise a potential pathogen and then react to counteract it. With basic incompatibility, the plant perceives some feature or stress associated with the invasion.

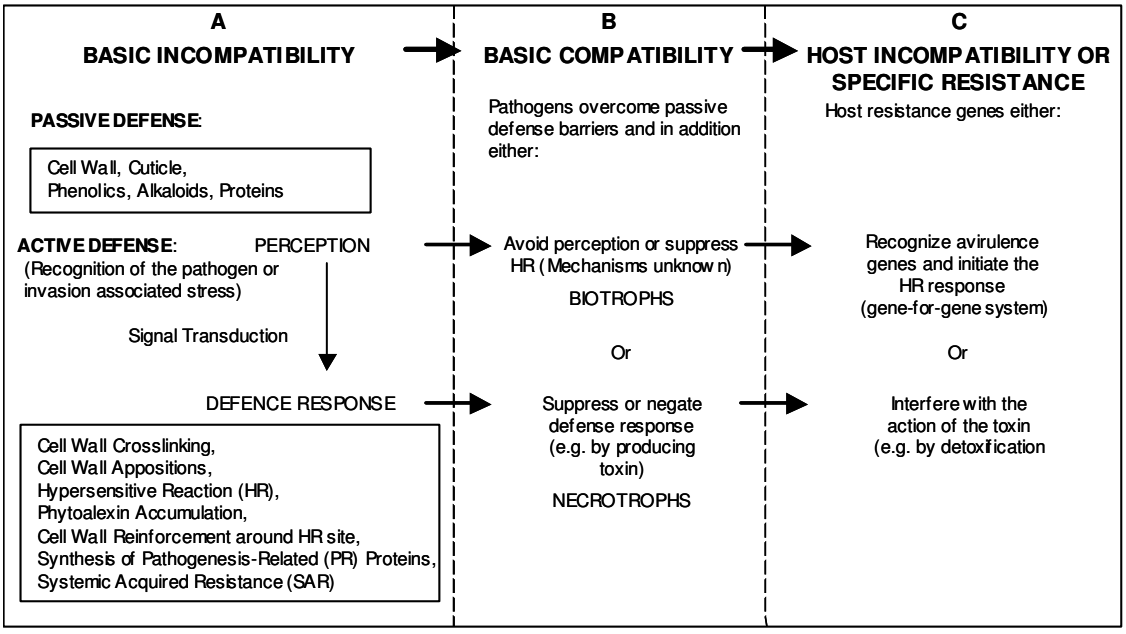


Fig. 2.1 Schematic representation showing mechanisms underlying resistance and disease in plant-fungal and host-pathogen interactions (Johal *et al*., 1995).

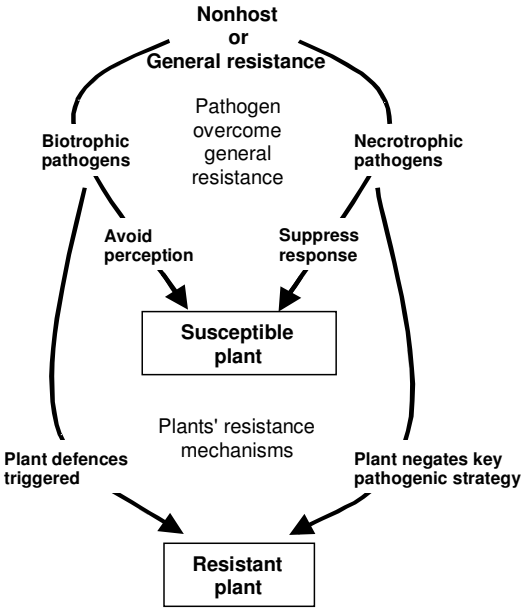


Fig. 2.2 Simplified illustration of plant-pathogen interaction (Modified from Johal *et al.*, 1995).

This leads to the release of signalling compounds that are responsible for the induction of the defence response (Clarke *et al.*, 1992; Ebel and Scheel 1992; Ebel and Cosio, 1994; Nurnberger *et al.*, 1994). With specific resistance against a biotrophic pathogen, a gene-for-gene interaction takes place whereby a corresponding gene product in the plant and the pathogen interact to determine incompatibility. In the absence of the reistance gene the HR is not triggered and the plant is susceptible to the pathogen (Flor, 1971).

Inducible defences only come into play if the plant is able to recognise the potential attacker and then activates the proper mechanisms to counteract it. Elicitors are involved in the recognition process between the plant and its invaders. Non-race-specific elicitors are responsible for the general or non-host resistance (Fig. 2.2) (Hahn, 1996). The gene-for-gene interactions, on the other hand, are responsible for specific or incompatible plant-pathogen interactions (Flor, 1971).

Pathogen-derived elicitors have been comparatively well characterized, but some insect derived elicitors of plant defences have only recently been identified. The first resistance/recognition gene that affected insects was a *Mi* gene from tomato. This *Mi* gene confers resistance to both root knot nematodes (Milligan *et al.*, 1998) and the potato aphid, *Marcrosiphum euphorbiae* (Rossi *et al.*, 1998). The wheat plant's ability to distinguish between mechanical wounding and RWA infestation is indicative of an elicitor, possibly in the aphid's saliva or stylet-sheath, which is responsible for inducing defence mechanisms including the expression of the PR-proteins (Kessler and Baldwin, 2001; Schmelz *et al.*, 2001).

Other insect elicitors include those isolated from Lepidopteran's oral secretions that elicit a systemic release of volatile plant compounds (Paré and Tumlinson, 1999). These volatile compounds attract natural enemies of herbivores such as parasitoid wasps and predatory mites (Dicke *et al.*, 1999). However, unlike pathogens, very few elicitors have been identified, which are unique to insect-plant recognition (Inbar *et al.*, 1998).

The identification of other insect-specific elicitors would provide an invaluable tool to investigate plant responses to herbivores, and thereby improve our understanding of the interplay between plant defences and pests. As mentioned, it has been shown that oligosaccharides, formed when β-1,3-glucanase hydrolyses β-1,3-glucans in the cell wall of pathogenic fungi, are responsible for eliciting the attack against the specific fungus (Keen and

Yoshikawa, 1983). Furthermore, glucanases are one of the groups of PR-proteins expressed at much higher levels in the resistant than in the susceptible plants when infested with the RWA (Van der Westhuizen *et al*., 1998a). But where does β-1,3-glucanases play a role in the fight against *Diuraphis noxia*?

2.4.1. Plant Signalling

Downstream of the perception of the pathogen is the activation of pre-existing protein kinases, phosphatases and ion fluxes including changes in Ca^{2+} concentration (Grant and Mansfield, 1999). A further rapid accumulation of reactive oxygen intermediates and nitric oxide is one of the earliest events in the HR (Delledonne *et al*., 2002) and as part of the HR, the PR genes are activated in the surrounding tissue (Hancock *et al*., 2002).

Jasmonic acid, salicylic acid (SA) and ethylene are central players in mediating responses to pathogens and wounding. Jasmonate is usually associated with wounding pathways, whereas salicylate is often thought to function in pathogen responses. However, there is ample evidence to show that wound signalling pathways sometimes function independently of jasmonate, and likewise, that there are salicylate-independent responses to pathogens (Fig. 2.3) (Pieterse and Van Loon, 1999). Mohase and Van der Westhuizen (2002) found that SA accumulation is differentially induced in RWA resistant wheat after RWA infestation. This elevated level could be responsible for the signalling mechanisms for the elevated downstream defence responses or for inducing systemic acquired resistance (SAR) (Fig. 2.3).

2.5. HYPERSENSITIVE RESPONSE (HR)

Hypersensitive cell death is a common resistance mechanism against microbial pathogens. Plants express resistance genes that trigger the HR through their specific interactions with pathogen avirulence genes (plant pathogen recognition and interaction) (Stotz *et al*., 1999). This gene-for-gene-interaction has generally been interpreted as a receptor-ligand model in which the avirulence protein binds to the corresponding resistance protein. Complex signal transduction pathways are then activated that involves changes in protein phosphorylation, production of reactive oxygen species and modification of ion fluxes. All of these reactions constitute the plant defence (Pontier *et al*., 1998; Bonas and Lahaye, 2002).

During the HR a necrotic lesion is formed around the initial site of pathogenic attack. The pathogen is restricted to this zone of cells because of a very intense induced defence response

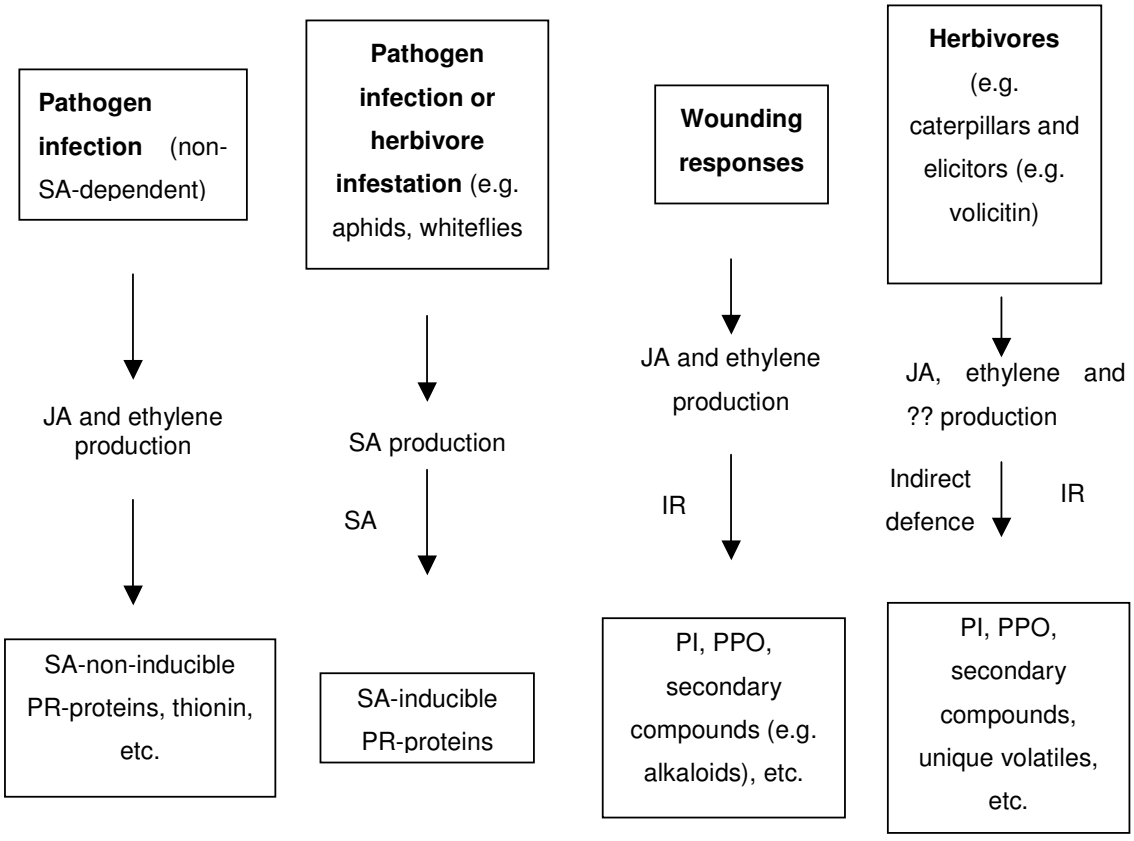


Fig 2.3 Simplified model showing signalling pathways associated with induced resistance to pathogens and herbivores. ?? denotes an unknown endogenous signal for elicitation. IR, induced resistance; PI, proteinase inhibitors and PPO, polyphenol oxidase. Adapted from Pieterse and Van Loon (1999).

(Lamb *et al.*, 1989). These defences can include the accumulation of derivatives of the phenyl-propanoid pathway (Fritig *et al.*, 1972; Legrand *et al.*, 1976; Legrand, 1983) and the accumulation of low molecular mass components like phytoalexins (Dixon *et al.*, 1983; Darvill and Albersheim, 1984; Ebel, 1986). The cell wall also plays a major role in the HR reaction. Changes include cell wall thickening and reinforcement by deposition of various macromolecules such as carbohydrates (callose), proteins (Bradley *et al.*, 1992) and hydroxyproline-rich glycoproteins (Varner and Lin, 1989), aromatic polymers such as lignin, lignin-like material, or other yet undefined cell wall bound phenolics (Vance *et al.*, 1980; Matern and Kneusel, 1988).

These necrotic spots are localised at the site of infection. However, systemic distribution of a signal causes the plant to develop resistance responses throughout the whole plant. This can be seen in the smaller lesions and greater restriction of the infection if inoculated by the same or other unrelated, but necrotizing pathogens (Ye *et al.*, 1989). In the case of pathogens, this overall resistance is known as systemic acquired resistance (SAR) (Pontier *et al.*, 1998).

The HR also leads to the induction of numerous plant genes encoding pathogenesis-related (PR) proteins such as β-1,3-glucanases, chitinases and PR proteins with unknown functions (Kombrink and Somssich, 1995). These findings were very important in understanding the interaction between the host and its pathogen. The so-called PR proteins were to become the keyword in any plant reaction to stress situations and not only with pathogens.

The HR associated with plant-insect interactions have not yet been studied so intensively as in the case with pathogens. However, some reports have shown that such insect resistance mechanisms do exist. Fernandes (1998) showed that the HR is responsible for the resistance of legume plants against a galling insect (Diptera, Cecidomyiidae) and, in *Brassica*, the HR lead to the killing of the eggs of *Pieres* butterflies (Shapiro and Devay, 1987). The involvement of HR in defence mechanisms against insects in these plant families (legumes and *Brassica*) suggests that such insect resistance mechanisms might be taxonomically widespread (Stotz *et al.*, 1999).

2.5.1. Pathogenesis-Related (PR) proteins

PR protein accumulation represents a major quantitative change in the soluble proteins during the activation of defence responses to pathogens or other stress conditions, which include insects (Cutt and Klessig, 1992; Stintzi *et al.*, 1993a; Van Loon *et al.*, 1994).

These PR-proteins are characterized by:

(a) Their acidic nature (Van Loon, 1976; Gininazzi *et al.*, 1977);

(b) Their resistance to the action of enzymes of endogenous or exogenous origin (Van Loon, 1982; Stintzi *et al.*, 1993a);

(c) Their location in compartments such as the vacuole, the cell wall and/or the apoplast (Payne *et al.*, 1989);

(d) Their low molecular weight (8-50 kDa).

With only a few exceptions, these PR-proteins are all monomers and often, but not always, have extreme isoelectric points (Stintzi *et al.*, 1993a). The extreme isoelectric points make them stable where they are secreted into the acidic intercellular space (Van Loon, 1985).

PR proteins, known from many plant species, can be classified into 17 families (Table 2.1). The sequence similarities, serologic or immunologic relationships and enzymatic properties are the basis of this classification (Van Loon *et al.*, 1994).

2.5.1.1. β-1,3-Glucanases

A large amount of evidence has been provided that β-1,3-glucanase expression and activity can increase in plants under different circumstances ranging from pathogen attack (Beerhues and Kombrink, 1994), elicitor treatments (Mauch *et al.*, 1984; Kombrink and Hahlbrock, 1986; Beerhues and Kombrink, 1994) to the spraying of the plants with a fungicide (Siefert *et al.*, 1996). This increase also takes place in plants infested with aphids like barley, which had a large increase in activity with birdcherry-oat aphid infestation (Forslund *et al.*, 2000). β-1,3-glucanase, in combination with chitinase, has been shown to destroy the fungus and to inhibit fungal growth in non-host plants (Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988b; Sela-Buurlage *et al.*, 1993).

Based on comparisons between deduced amino acid sequences of tobacco β-1,3-glucanases they have been grouped into classes I, II and III (Table 2.1) (Linthorst *et al.*, 1990; Payne *et al.*, 1990).

Most of the glucanases in plants are endo-β-1,3-glucanases producing oligomers of 2,6 glucose units from callose (β-1,3-D-glucan) (Stintzi *et al.*, 1993a). The exo-β-1,3-glucanases successively hydrolyse β-D-glucose units from the non-reducing ends of β-1,3-D-glucans,

Table 2.1. Pathogenesis-related (PR-) proteins of tobacco (Van Loon *et al*., 1994).

Family	Group	Molecular weight	Properties
PR-1	1a 1b 1c	15.5 to 19 kDa	Antifungal activity
PR-2	2 N O	25 to 40 kDa	Class I, II and III endo-glucanases
PR-3	3 P Q	27 to 33 kDa	Class I, II, III, IV, V, VI and VII endo-chitinases
PR-4	R	13 to 19 kDa	Antifungal, *win*-like proteins, endochitinase activity, similar to prohevein C-terminal domain.
PR-5	S	23 to 26 kDa	Thaumatin-like proteins, zeamatins, permeatins similar to alpha-amylase/trypsin inhibitors, Osmotin: salt stress, other functions include antifungal activity in tobacco, maize, and other grain species.
PR-6	Tomato inhibitor 1	6-13 kDa	Protease inhibitors
PR-7	Tomato P		Endoproteases
PR-8	Cucumber chitinase		Class III chitinases, chitinase/lysozyme
PR-9	Lignin-forming peroxidase		Peroxidases, peroxidases-like proteins
PR-10	Parsley PR-1		Ribonucleases, Bet v 1-related proteins
Pr-11	Tobacco class V chitinase		Endochitinase activity

PR-12	Radish Ps-AFP3		Plant defensins
PR-13	*Arabidopsis* THI2.1		Thionins
PR-14	Barley LTP 4		Nonspecific lipid transfer proteins (ns-LTPs)
PR-15	Barley OxOa (germin)		Oxalate oxidase
PR-16	Barley OxOLP		Oxalate-oxidase-like proteins
PR-17	Tobacco PRp27		Unknown

releasing glucose molecules (Bielka *et al*., 1984). *In vitro*, laminarin, which is also a glucan, is used to quantify the expression and activity of the glucans in plant protein extracts (Fink *et al*., 1988).

Callose (a β-1,3-glucan) which is the main substrate for β-1,3-glucanases in plant tissue, like cellulose, is produced by transmembrane proteins. The specific mechanism responsible for the production of callose is, however not completely understood (Kudlicka and Brown, 1997). It seems that both callose and cellulose are synthesized within a single enzyme complex. Callose only becomes the predominant product during cell disruption (Jacob and Northcote, 1985; Delmer, 1987). The glucose molecule, which is the substrate, is thought to be delivered to the enzyme complex at the cytoplasmic face of the plasmamembrane. The fibrillar layers of callose are deposited in the extra cellular space, as glucan synthase complexes move through the plane of the lipid bilayer (Kudlicka and Brown, 1997). Deposition of callose has been implicated in numerous studies as a structural resistance mechanism by plants against the attempt penetration by fungi (Aist, 1976; Bayles *et al.*, 1990; Benhamou, 1992; Stanghellini *et al.*, 1993). Callose has also been shown to be a major part of pollen tubes and grains (Ferguson *et al.*, 1998) and might play a role in plugging sieve tube cell plates (Abeles and Forrence, 1970). It is also a major structural component of fungal cell walls (McNeil *et al.*, 1984). β-1,3-glucanases, which are responsible for the depolimerisation and mobilization of the β-1,3-glucans, can therefore play a definite role in the mobilization of the glucans to the site of infection and therefore activate the whole resistance mechanism (Hinch and Clarke, 1982; Schmele and Kauss, 1990).

Glucanases have been found to play a role in a variety of physiological processes. This include cereal germination (Stuart *et al.*, 1986; Hoj *et al.*, 1989), hypocotyl and coleoptile growth (Goldberg, 1980; Huber and Nevins, 1980), regulation of phloem transport through callose mobilization (Clarke and Stone, 1962; Abeles and Forrence, 1970; Wong and Maclachlan, 1980), flower development (Neale *et al.*, 1990; Ori *et al.*, 1990), pollen tube growth (Roggen and Stanley, 1969) and fruit maturation (Hinton and Pressey, 1980). It also plays major roles in defence against pathogens (Mauch *et al.*, 1988a and b; Vögeli *et al.*, 1988).

Another potential role of these β-1,3-glucanases is the conversion of β-glucan polymers into soluble forms capable of triggering the plant defence response (Darvill and Albersheim, 1984). The first fungal elicitors identified were oligo-β-glucans, later characterized as hepta

-β-glucosyl fragments which are composed of linear β-1,3-linked glucans with occasional 6-linked branching residues (Hahn *et al.*, 1989). The receptors that bind these elicitors are possibly located in the plasma membrane which recognize hepta-glucosides and distinguish them from glucans with β-1,3 and β-1,6-linkages present in healthy plant cells (Ayers *et al.*, 1976; Darvill and Albersheim, 1984; Sharp *et al.*, 1984; Cosio *et al.*, 1990).

The possibility of hepta-β-glucosyl fragments forming during the probing of the RWA could also explain the triggering of the hypersensitive response and PR-protein expression. The β-1,3-glucanase could then in turn mobilize β-1,3-glucans to the probing site blocking the phloem and subsequently also the flow of sap to the feeding aphid. This could explain why aphids switch from phloem ingestion to non-phloem ingestion on resistant wheat compared to susceptible plants (Webster *et al.*, 1993).

2.5.1.1.1. Localisation

Studies on the subcellular localisation of hydrolytic enzymes *in planta* can provide important information about the role of the accumulated hydrolytic enzymes during active defence.

Mauch and Staehelin (1989) and Mauch *et al.*, (1992) investigated the subcellular localisation of chitinase and β-1,3-glucanase in ethylene-stressed bean leaves and proposed a model outlining the possible implication of these hydrolytic enzymes in defence and recognition events during host-parasitic interactions. In this model the cell wall-localised β-1,3-glucanases are involved in releasing signal molecules from the walls of the invading pathogens. The high concentrations of chitinase and β-1,3-glucanases in the vacuole act as the last line of defence to be released when the attacked host cells lyse. This would flood the pathogen with high concentrations of hydrolysing enzymes making it impossible to adapt.

Benhamou *et al.*, (1989) examined the spatial localisation of β-1,3-glucanases in vascular wilt fungi infected tomato plants. In the susceptible plants, labelling was only present on cell walls of invaded cells and cells closely neighbouring fungal cells. The labelling density decreased dramatically if the host cell was not in direct contact with the invading pathogen. In the incompatible interaction, heavy labelling was observed in secondary thickenings of uncolonised xylem vessels. It would seem that in the resistant plants the accumulation of β-1,3-glucanases is an early event limiting the spread of the fungus, whereas in the susceptible plants it is as a result of successful tissue colonisation (Benhamou *et al.*, 1989).

The inducing elicitor could play an important role in the accumulation of the β-1,3-glucanases at different strategic sites (Van den Bulcke *et al*., 1989). This could explain different results obtained with different induction agents even though they seem to be similarly induced by chemical, hormonal or pathogenic elicitors (Boller, 1985).

An unique method whereby a β-1,3-glucanase was conjugated to colloidal gold was used to localise the deposition of callose, the substrate for β-1,3-glucanase in tobacco plants after infection with *Phytophthora parasitica* var. *nicotianae* (Benhamou, 1992). Callose was found at sites of attempted penetration in sieve tube members, plasmodesmata and intercellular spaces. Labelling also occurred on the outer cell wall of the invading pathogen. The localisation of callose on the plasmodesmata could play a role in decreasing the flow of nutrients to the pathogen (Benhamou, 1992). Higher β-1,3-glucanases could therefore increase the amount of available callose deposited on the plasmodesmata.

The antibody used in this study was produced in our department by X-M Qian (Van der Westuizen *et al*., 2002), it was also used by Hu and Rijkenberg (1998) to localise β-1,3-glucanase in *Puccinia reconditia* f.sp. *tritici*-infected wheat leaves. In these wheat plants labelling was found on the cell walls of guard cells, phloem elements and the secondary thickenings of xylem elements. The cell wall apposition, which is part of the resistance response to the fungal attack and the membrane-like structures adjacent to the aposition, was heavily labelled. The results were in accordance to Sock *et al*., (1990) and Wubben *et al*., (1992) with the labelling they found in plant pathogen interactions.

2.6. AIM

The main aim of the study was to learn more about the expression of β-1,3-glucanase during the resistance response of wheat to the RWA.

To achieve this, the objectives were:

- To study the effect of different resistance genes on the expression of β-1,3-glucanase on activity and protein levels to establish the universality of its expression.
- To study the expression of β-1,3-glucanase as affected by the Dn1 resistance gene on transcriptional level.

- To localise β-1,3-glucanase accumulation on subcellular level in order to shed more light on its possible defensive function.
- To establish any link between chloroplast survival in resistant plants on RWA infestation and β-1,3-glucanases.

CHAPTER 3

MATERIAL

AND

METHODS

3.1. PLANT MATERIAL

Triticum aestivum L. (wheat) seed of the susceptible Tugela cultivar, and the near isogenic resistant Tugela cultivars, containing the Dn1 (PI 137739), Dn2 (PI 262660) and Dn5 (PI 294994) resistance genes respectively, were obtained from the Small Grain Institute at Bethlehem. Wheat plants were grown in a sand soil mix (2:1) in 6 rows in pots (30 x 40 cm) in the greenhouse at a day temperature of 24°C (±2°C) and night temperature of 16°C (±2°C). Plants were infested at the two-leaf stage at the beginning of the third leaf stage. The plants were infested with aphids that were collected from plants containing breeding colonies, by scattering them evenly onto the plants (ca. 5 aphids/plant).

3.2. METHODS

3.2.1. Collection of intercellular washing fluids (IWF)

IWF was collected at different time intervals namely, 0, 6, 12, 24, 48 and 120 hours post infestation (hpi) (Rohringer *et al*., 1983). The second and third leaves were removed from both infested and uninfested resistant and susceptible plants by cutting them with a sharp razor. The leaves were cut into 7 cm pieces and rinsed with distilled water. The leaves was then vacuum infiltrated with 50 mM Tris(hydroxymethyl)aminomethane (Tris) (pH 7.8) containing 5 mM mercapto-ethanol, 0.5 mM phenylmethylsulphonylfluoride (PMSF) in a glass tube for 5 minutes using a water jet pump. Care was taken to release the vacuum very slowly to infiltrate the leaves with the buffer and limit the damage to the plant cells. The leaves were dried on paper and placed into centrifuge tubes containing a perforated disc at the bottom. The tubes were centrifuged at 500 *g* for 10 min and the IWF collected and frozen at -20°C. The whole procedure was repeated twice for each sample.

The collected IWF was used for β-1,3-glucanase activity determinations and Western blot analysis.

3.2.2. Determination of the protein concentration

The protein concentration of each sample was determined using the dye-binding assay technique of Bradford (1976). Bovine γ-globulin (Sigma) (0.5 $mg.ml^{-1}$) was used as standard.

The absorbance at 595 nm was read on the Bio-Rad Model 3550 microplate reader (Rybutt and Parish, 1982).

3.2.3. Malate dehydrogenase (MDH) activity

MDH activity was determined as a measure of cytosol contamination and was carried out according to Rohringer *et al*., (1983). The reaction mixture (1 ml) contained 3.75 mM 1,4-dithiothreitol (DTT), 7 mM $MgCl_2$, 0.25 mM NADH, 2.3 mM oxalacetic acid in 80 mM phosphate buffer (pH 7.5) and 20 µl IWF. The oxidation of NADH was followed at 340 nm for 1 min as an indication of malate production. Contamination of the apoplastic fluid was expressed as percentage MDH activity of the total (apoplastic and symplastic) MDH activity. The MDH activities of five random samples were measured.

3.2.4. β-1,3-glucanase activity

β-1,3-glucanase activity was assayed by measuring the rate of reducing sugar production from laminarin (Fink *et al*., 1988). The reaction mixture consisted of 500 µl 50 mM potassium acetate (pH 4.5) containing 2 $mg.ml^{-1}$ laminarin and 10 µl IWF. After incubation at 37ºC for 10 min, 500 µl of Somogyi reagent [0.4 g $CuSO_4$, 18 g Na_2SO_4, 2.4 g Na_2CO_3 and 1.2 g potassium tartrate in 100 ml distilled water (Somogyi, 1952)] was added. After cooling, 500 µl Nelson's reagent [5.3 g $(NH_4)_6Mo_7O_{24}$, 4.4 ml H_2SO_4, 0.64 g $Na_2AsO_4.7H_2O$ in 100 ml distilled water (Nelson, 1944)] was added. A blank in which the laminarin was omitted was used to zero spectrophotometer. The absorbance of the coloured product was read at 540 nm.

A standard curve (Fig. 4.1) relating the absorbance to known glucose concentrations was used to calculate the enzyme activities. The β-1,3-glucanase activity was expressed as mg glucose mg-1 protein min-1. β-1,3-glucanase activities were determined in triplicate. Two independent experiments were performed.

3.2.5. Sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots

3.2.5.1. SDS-PAGE gels

SDS-PAGE was performed using 12% (m/v) running and 6% (m/v) stacking gels (ratio of acrylamide to N, N-methylene-bis-acrylamide 30:1) according to Laemmli (1970) and a Bio-Rad minigel electrophoresis unit. After determining the protein concentrations, 20 µg protein

were precipitated with 9 volumes of acetone for 1 h at -20ºC. After centrifugation (3000 *g*), for 10 min, the pellets were dried at room temperature for 15 min. The protein was dissolved in sample buffer containing 0.0625 M Tris (pH 6.75), 2% (m/v) SDS, 5% (v/v) mercapto-ethanol, 10% (v/v) glycerol and 0.001% (m/v) bromophenol blue. The Low Range SDS-PAGE Molecular Weight Standards from Bio-Rad were used to estimate the molecular mass of the separated polypeptides. The gels were stained using the Coomassie staining method [0.2% (m/v) Coomassie Blue R250, 0.1% (m/v) Coomassie Blue G250, 7% (v/v) acetic acid, 50% (v/v) ethanol]. The gels were destained in 7% (v/v) ethanol, 43% (v/v) acetic acid.

3.2.5.2. Western Blots

Protein separated by SDS-PAGE was transferred to Hybond-C nitrocellulose membranes (Amersham) using a Bio-Rad Trans-blot SD semidry electrophoretic transfer cell. A 25 mM Tris (pH 8.3) transfer buffer containing 192 mM glycine and 20% (v/v) methanol was used for the transfer at 80V for 1 h.

After protein transfer the nitro-cellulose membranes were stained with Ponceau-S (Merck) to mark the molecular markers and then destained with TBS [10 mM Tris (pH 7.9), 150 mM NaCl] for 5 min. The membrane was blocked with 8% (m/v) fat free milk powder in TBS for 1 h, incubated in the primary antibody (Van der Westhuizen *et al.*, 2002) diluted 1:9000 in TBS for 1 h and washed 3 times in TBST buffer [TBS with 0.1% (v/v) Tween-20]. The blot was then probed with goat anti-rabbit IgG (Sigma) at a 1:8500 dilution in TBS for 1 h and washed 3 times, 5 min each with TBST and TBS respectively.

The cross-reacting proteins were identified by the enzymatic cleavage of the phosphate group of 5-bromo-4-chloro-3-indolyl phosphate (BCIP), using nitro blue tetrazolium (NBT) as a stain enhancer in an epitope-staining buffer [100 mM Tris (pH 9.5), 100 mM sodium chloride, 5 mM magnesium chloride, 0.3 $mg.ml^{-1}$ NBT and 0.2 $mg.ml^{-1}$ mM BCIP] (Blake *et al.,* 1984). Differences in the band intensities were compared visually.

3.2.6. RNA analysis

3.2.6.1. RNA extraction

Leaves from infested and uninfested resistant and susceptible plants were collected at 0, 12, 24, 48 and 120 hpi and frozen in liquid nitrogen. Total RNA was isolated from this material using a modified method of Chomczynski and Sacchi (1987). The frozen plant material (0.1

to 0.2 g) was ground in 0.8 ml extraction buffer (4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% (m/v) sarcosyl and 0.1 M 2-mercapto-ethanol). After grinding, 0.5 ml of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8) was added. The mixture was vortexed and centrifuged at 6000 *g* for 3 min. The upper phase was transferred to a new sterile eppendorf and 50 µl of 3 M sodium acetate (pH 4.0), 500 µl acidic phenol and 100 µl chloroform were added. The tubes were inverted 3 times and centrifuged at 6000 *g* for 5 min. The upper phase was again transferred to a new eppendorf and 500 µl chloroform was added. The tube was vortexed and centrifuged at 6000 *g* for 5 min. The upper phase was precipitated with an equal volume of 2-propanol for 30 min. at –20ºC after which it was centrifuged at 6000 *g*, the pellet washed with 70% (v/v) ethanol, dried and resuspended in 100 µl of sterile water.

The RNA concentration was calculated by reading the sample absorbance at 260 nm and using RNA absorbance of 1 OD as 40 mg RNA (Sambrook *et al*., 1989). To assess the quality of the RNA 1 µg was loaded onto a 0.8% (m/v) agarose gel made in TAE buffer [40 mM Tris-acetate, 1mM EDTA, pH 8.0) and separated at 80V for 1 h.

3.2.6.2. RNA separation and transfer

Northern blots were performed to investigate the expression of β-1,3-glucanase on mRNA level. A total of 20 µg RNA for each time point was freeze dried and dissolved in 18 µl RNA buffer [0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS), 50% (v/v) formamide, 6.5% (v/v) formaldehyde, 50 $\mu g.ml^{-1}$ ethidium bromide]`. These RNA samples were denatured at 65ºC for 10 min. and cooled on ice. Before loading on a 1% (m/v) agarose-formaldehyde gel [1% (m/v) agarose, 400 mM formaldehyde, 20 mM MOPS, pH 7.0, 8 mM sodium acetate, 1 mM EDTA], 2 µl RNA loading buffer [50% (v/v) glycerol, 1 mM EDTA pH 8.0, 0.25% (m/v) bromophenol blue] was added to each sample. The samples were separated at 50V for 1 h.

The separated RNA was transferred to a Hybond Nylon^{+} membrane by the alkaline transfer method described by Chomczynski (1992) using an alkaline transfer buffer [3 M NaCl, 8 mM NaOH]. The RNA was transferred for 2 h. The membrane was neutralized in 0.2 M-phosphate buffer (pH 8) for 5 min and dried for 20 min at 70ºC.

RNA separation and quality was assessed by staining of the membrane with 0.02% (m/v) methylene blue dissolved in 0.3 M NaOAC, pH 5.5 for 5 min. The membranes were destained with water. The RNA standards were marked and the membrane was totally destained in 0.2X

sodium citrate buffer (SSC) [3 mM NaCl and 3 mM sodium citrate (pH 7)] containing 1% (m/v) SDS for 15 min. at room temperature.

Water was treated with 0.1% diethyl pyrocarbonate (DEPC) overnight and then autoclaved for 20 min at 121°C. All the solutions used for RNA blots were made up in DEPC treated water.

3.2.6.3. β-1,3-Glucanase probes

i) A soybean β-1,3-endoglucanase cDNA clone was obtained from Dr. Yoji Takeuchi from Japan (Takeuchi *et al*., 1990). This β-1,3-glucanase was isolated from an expression library synthesized from mRNA of ethylene treated soybean seedlings. It codes for a 33.5 kDa mature β-1,3-glucanase protein with a 2.5 kDa signal peptide responsible for targeting it to the intercellular space. The gene was cloned into the *Eco*R1 site of the pBluescriptKS plasmid, which was named pEG488.

ii) A GII barley β-1,3-glucanase cDNA clone was obtained from Simon Rutten from Australia (Hoj *et al*., 1989). This β-1,3-glucan endohydrolase isoenzyme II was isolated from a cDNA library synthesized from mRNA of excised scutella of germinating barley seeds. The clone, which codes for a 32 kDa protein with a pI of 10, was subcloned into the *Nde*1 and *Sac*11 restriction sites of the plasmid pET3aHT. The fragment also has an internal SacII restriction site so that when digested with *Nde*I and *Sac*II, two fragments (400 bp and 650 bp) forms.

To prepare the β-1,3-glucanase probes, plasmids containing the soybean and barley DNA probes were transformed into competent *Escherichia coli* cells according to Inoue *et al.*, (1990). These transformed *E. coli* cells were inoculated into 100 ml LB medium [50μg.ml^{-1} ampicilin, 1% (m/v) tryptone, 0.5% (m/v) yeast extract, 1% (m/v) NaCl (pH 7)] and grown overnight at 37ºC. Plasmid DNA was extracted according to Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). An aliquot of 20 μg plasmid was digested with the corresponding enzymes according to manufacturer's specifications (Boehringer). The digested plasmids were separated on an agarose gel and the β-1,3-glucanase probe fragments were excised from the gel. The DNA was isolated from the gel using Geneclean (Southern Biotechnologies) according to the manufacturers protocol. The concentrations of the isolated DNA probes were determined by measuring their absorbance at 260 nm and using DNA absorbance of 1 OD as 50 mg RNA (Sambrook *et al*., 1989). The probes were labelled with [α-^{32}P]dATP (ICN) using the Megaprime DNA labelling system (Amersham) according to

the manufacturer's directions. These were then used to probe the RNA blotted onto the membrane.

3.2.6.4. Northern blot hybridisation

The membrane prepared in 3.2.6.2. was placed in a roller tube previously baked at 250ºC overnight. To this tube 25 ml prehybridisation buffer [50% (v/v) formamide, 5X SSC (0.75 M NaCl and 75 mM sodium citrate (pH 7.0)], 5X Denhardts [0.1% (m/v) bovine serum albumin (BSA), 0.1% (m/v) Ficoll, 0.2% (m/v) polyvinylpyrrolidone (PVP), 0.5% (m/v) SDS, 50 $\mu g.ml^{-1}$ salmon sperm DNA] was added and incubated at 42ºC for 1 h. The probe was denatured at 95ºC for 5 min and then placed on ice. The probe was resuspended in pre-hybridisation solution, added to the blot and hybridised overnight at 42ºC. After the incubation period, the blot was washed twice in washing buffer 1 (2X SSC [300 mM NaCl, 30 mM sodium citrate (pH 7)], 0.1% (m/v) SDS) for 5 min each at room temperature. The blot was transferred to a new roller tube and washed in washing buffer 2 (0.2X SSC [3 mM NaCl, 0.3 mM sodium citrate pH 7], 0.1% (m/v) SDS) for 15 min. at 65ºC. The membrane was sealed in a plastic bag and exposed to an X-ray film.

The membrane was stripped after the soybean glucanase probe hybridisation and reprobed with the GII barley glucanase probe. The membrane was stripped by adding boiling 0.1% (m/v) SDS and incubating it for 30 min. after which it was washed in 2X SSC (McCaughern-Carucci, 1997).

3.2.7. Immuno-localisation of β-1,3-glucanase in wheat leaves

3.2.7.1. Sampling and preparation of leaf sections

Leaves from infested and uninfested susceptible (Tugela) and resistant (Tugela DN) wheat plants were collected at time intervals, 0, 48 and 336 hpi respectively. At each time interval the lower part of the leaves where the aphids fed were collected, and fixed using the following two protocols. The first group of leaves were cut into 3 X 15 mm pieces and fixed with 3% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0), for 1 h at room temperature and then rinsed in 0.1 M sodium phosphate buffer (pH 7.0). The tissue pieces were post-fixed for 1 h with 1% (m/v) osmium tetroxide (OsO_4) in 0.1 M sodium phosphate buffer (pH 7.0) and then dehydrated in an ethanol series [50%, 70%, 95% and 100% (v/v)] for 15 min each. It

was then infiltrated for 1 h with a 100% (v/v) ethanol - LR White mixture (1:1), left overnight in 100% (v/v) LR White and polymerized at 50ºC for 12 h.

With the second group the same protocol was followed except the osmium tetroxide fixation was omitted.

Ultra thin sections were cut using a glass knife and collected on 400 mesh nickel grids (Agar Scientific) for immunocytochemical processing. Thirty sections from two leaf samples were examined.

3.2.7.2. Immunogold labelling

Nickel grids containing the sections of the osmium tetroxide fixed group (Group 1)(3.2.6.1) were first incubated in 4% (m/v) aqueous sodium metaperiodate for 30 min and then washed with distilled water. The labelling protocol that follows was similar for group 1 and 2. Nickel grids containing the sections were transferred to one drop of 10 mM phosphate-buffered saline (PBS) [10.4 mM Na_2HPO_4, 3.2 mM KH_2PO_4, 150 mM NaCl, pH 7.4], containing 0.2% (m/v) polyethylene glycol (PEG) 20000) for 5 min at room temperature. The sections were then incubated in a drop of blocking solution consisting of PBS (pH 7.4), 1% (m/v) BSA, 0.05% (v/v) Tween-20, 0.2% (m/v) sodium azide (NaN_3) for 30 min at room temperature. The grids were then transferred to a drop of primary antibody (antiserum against β-1,3-glucanase) diluted 1:1200 in 10 mM PBS (pH 7.4), 1% (m/v) BSA in a moist chamber overnight at 4ºC. After washing in a series of drops of washing solution [PBS, pH 7.4, containing 1% (m/v) BSA and 0.05% (v/v) Tween-20], the grids were transferred into a drop of colloidal gold (15 nm)-conjugated goat anti-rabbit IgG (GAR-gold antibody) diluted 1:20 in PBS (pH 7.4) containing 1% (m/v) BSA for 1 h at room temperature. The grids were finally washed with PBS (pH 7.4), fixed in 1% (v/v) glutaraldehyde for 2 min, rinsed with double distilled water and counter-stained with 5% (m/v) uranyl acetate, 0.1% (m/v) lead citrate (Reynolds, 1963). The sections were viewed with a Philips (Tokyo, Japan) SM 100 Transmission Electron Microscope at 80 kV.

The specificity of the immunogold labelling was determined by incubation of the sections with pre-immune serum (1:1200 in PBS) instead of the primary antibody and by incubating the sections only in PBS-BSA buffer without added primary antibody or pre-immune serum.

3.2.7.3. Assessing β-1,3-glucanase epitope stability

Proteins were extracted from 48 h infested resistant plants by grinding the tissue in liquid nitrogen and adding extraction buffer [50 mM Tris (pH 7.5), 2 mM EDTA, 10 mM mercapto-ethanol and 2 mM PMSF]. The extract was centrifuged at 5000 *g* for 15 min. The supernatant was transferred to a new tube and used for protein analysis. The protein concentration was determined (3.2.2.) and a dilution range from 10 to 30 µg was separated on a SDS-PAGE gel (3.2.5.1.). The proteins were transferred to a nitro-cellulose membrane using the Bio-Rad Trans-blot SD semidry electrophoretic transfer cell. The first membrane was treated with 1% (m/v) osmium tetroxide (OsO_4) in 0.1 M sodium phosphate buffer, pH 7.0 for 1 h at room temperature. The control membrane was left in 0.1 M sodium phosphate buffer lacking osmium tetroxide. After the incubation period, both membranes were washed with TBS and a Western blot (3.2.5.2.) was performed on the membranes to assess any differences in epitope marking after osmium tetroxide treatment.

3.2.7.4. Quantification of labelling

The density of labelling associated with plant structures was calculated and expressed as the number of gold particles per square micrometer (particles.μm^{-2}). The densities were determined by counting the particles per cm^2 on a photographic enlargement. The enlargement of the photograph was taken into account converting gold particles per cm^2 to gold particles μm^2. Approximately twenty of the areas per sample were counted and the average and standard deviation determined.

3.2.8. Chloroplast proteins investigation

3.2.8.1. Chloroplast fractionation

Figure 3.1 is a schematic illustration of the purification protocol used for the chloroplast fractionation. Wheat plants were grown and infested as described (3.1.). The plants were placed in the dark for 16 h to limit the amount of the starch formed. Leaves of infested and uninfested resistant and susceptible plants were collected at 120 hpi. The leaves were homogenized with an Ultra-Turrax in extraction buffer (35 mM mannitol, 5 mM EDTA, 1 mM mercapto-ethanol, 5 mM Tris pH 8) at a ratio of 1 g plant material to 4 ml extraction buffer. Hereafter the homogenate was passed through four layers of cheese cloth. The extract was centrifuged at 50 *g* for 10 min and the supernatant centrifuged at 1500 *g* for 15 min to pellet the chloroplasts. The chloroplast pellet was resuspended carefully with a soft brush in

cold wash buffer (35 mM mannitol, 25 mM EDTA, 5 mM Tris pH 8). The suspension was loaded on a sucrose gradient (Fig. 3.1).

A discontinues sucrose gradient was prepared by layering two different sucrose concentrations [30% (m/v) and 52% (m/v) sucrose in washing buffer] in an ultra-centrifuge tube. The sucrose gradient was centrifuged at 12500 *g* in an ultra-centrifuge for 60 min. The chloroplast band was removed with a pasteur pipet and diluted ten times with washing buffer after which it was centrifuged at 1500 *g* for 15 min. The pellet was then resuspended in the washing buffer and again centrifuged at 1500 *g* for 15 min. For the preparation of the stromal extract the chloroplasts were resuspended in a hypotonic buffer (10 mM Tris-HCl pH 8) at 4ºC and left for 10 minutes to ensure complete lysis. The membranes were pelleted by centrifugation at 40000 *g* for 20 min. The supernatant consisted of all the stromal proteins (Fig. 3.1).

The membranes were resuspended in 5 mM Tris buffer (pH 8) and treated with 0.1% (v/v) Nonidet for 20 min. The solution was centrifuged at 40000 *g* for 20 min and the supernatant stored at –80ºC. This treatment was included to extract possible membrane bound proteins (Fig. 3.1).

These fractions were used for β-1,3-glucanse activity and Western blot analysis.

3.2.8.2. β-1,3-Glucanase activity of chloroplast fractions

The different chloroplast fractions obtained in (3.2.8.1.) were used to determine the β-1,3-glucanase activity as described (3.2.4.). To eliminate possible artefacts due to differences in reducing sugars present in the chloroplasts, the reducing sugar levels were determined by omitting laminarin as a blank. This absorbance value was subtracted from that of the sample with laminarin and this value was used to calculate the glucanase activity of each sample.

3.2.8.3. Western blots of chloroplast fractions

Proteins obtained from the chloroplast fractions were prepared by precipitating proteins from the supernatants (Fig. 3.1) with nine volumes of acetone at 4ºC for 1 h. The samples were centrifuged, the pellets washed twice with 80% (v/v) acetone, dried and dissolved in water. The protein concentration was determined (3.2.2.) and thirty microgram protein was used in the Western blots (3.2.5.2.).

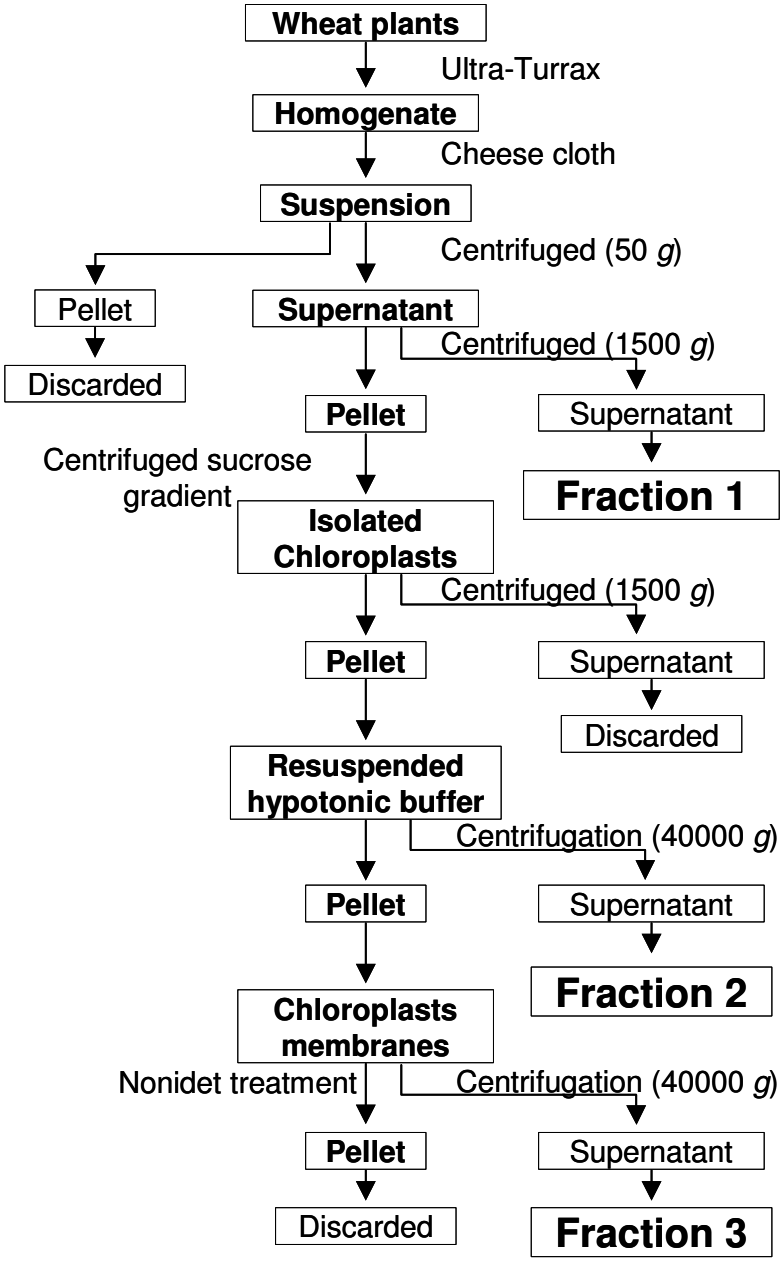


Fig. 3.1 Schematic illustration of the chloroplast fractionation steps. Fractions 1, 2 and 3 represents the fractions collected for analysis.

CHAPTER 4

RESULTS

4.1. β-1,3-GLUCANASE EXPRESSION

4.1.1. Purity of the intercellular wash fluid (IWF)

The apoplast plays a central role in the plant's defence response. The IWF constitutes the apoplast liquid and contains, amongst others, extracellular proteins, many of which may be PR proteins. Cell damage will result in the cytosolic contamination of the IWF in which case the IWF will contain intracellular proteins, which could affect the estimation of intercellular enzyme activities. Malate dehydrogenase (MDH) is a cytoplasmic enzyme and its activity in the IWF would give an indication of the level of cytoplasmic contamination. During all our experiments, the MDH activity was less than 0.1% of the total MDH activity, indicating very little contamination of the IWF.

4.1.2. The activation of β-1,3-glucanase activity

The β-1,3-glucanase activity determination involves the estimation of the rate at which reducing sugars (e.g. glucose) are released from the substrate, laminarin (Fink *et al.,* 1988). Figure 4.1 B represents a standard curve relating the absorbancies of the coloured product formed to different glucose concentrations, during the determination of reducing sugars using the Nelson and Somogyi method (Nelson, 1944; Somogyi, 1952). The linear region of the curve was used in the calculations of the β-1,3-glucanase activities (Fig. 4.1 A).

RWA infestation did not cause any significant induction of the β-1,3-glucanase activity in susceptible (cv. Tugela) plants during two separate experiments (Fig. 4.2 IA and IIA). β-1,3-glucanase activity was markedly induced after 48 h of infestation in resistant plants containing the Dn1 (Fig. 4.2 IB and IIB) and Dn2 (Fig. 4.2 IC and IIC) resistance genes. In the first experiment (Fig. 4.2 IB and C), after 120 h of infestation, the activities in Dn1 and Dn2 resistant plants were almost two fold higher compared to uninfested plants. In the second experiment, the induced activities were comparable to that of the first (Fig. 4.2 II B and C).

RWA infestation had very little effect on the β-1,3-glucanase activity of resistant plants containing the Dn5 resistance gene (Fig. 4.2 I and II D).

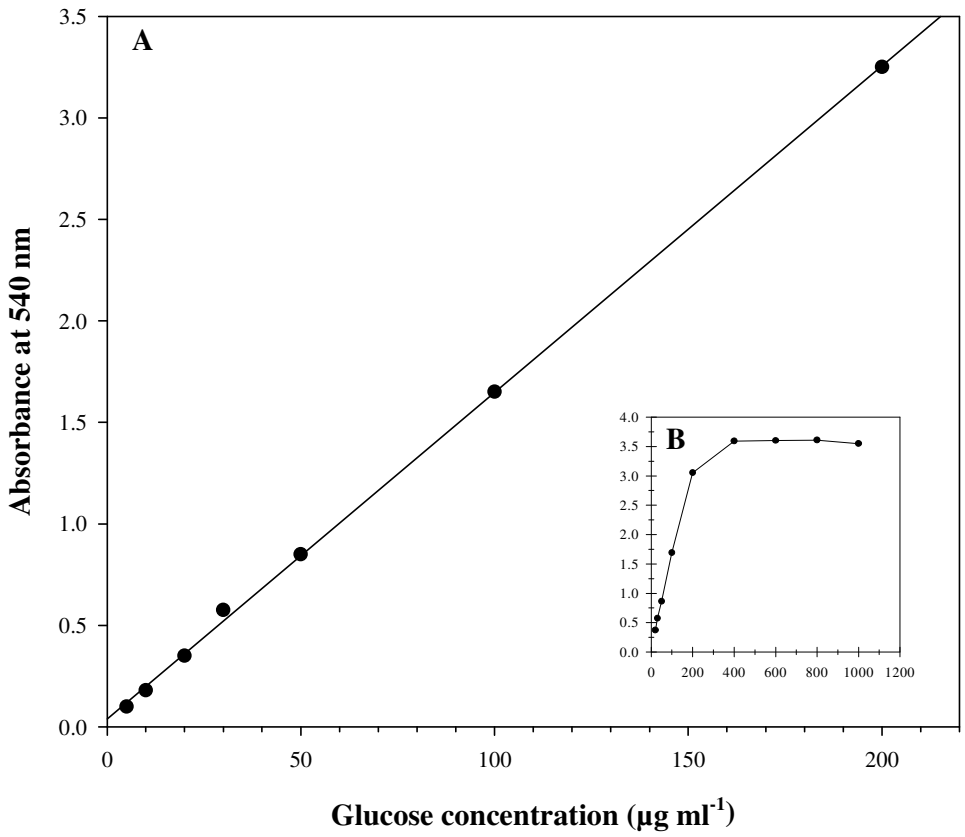
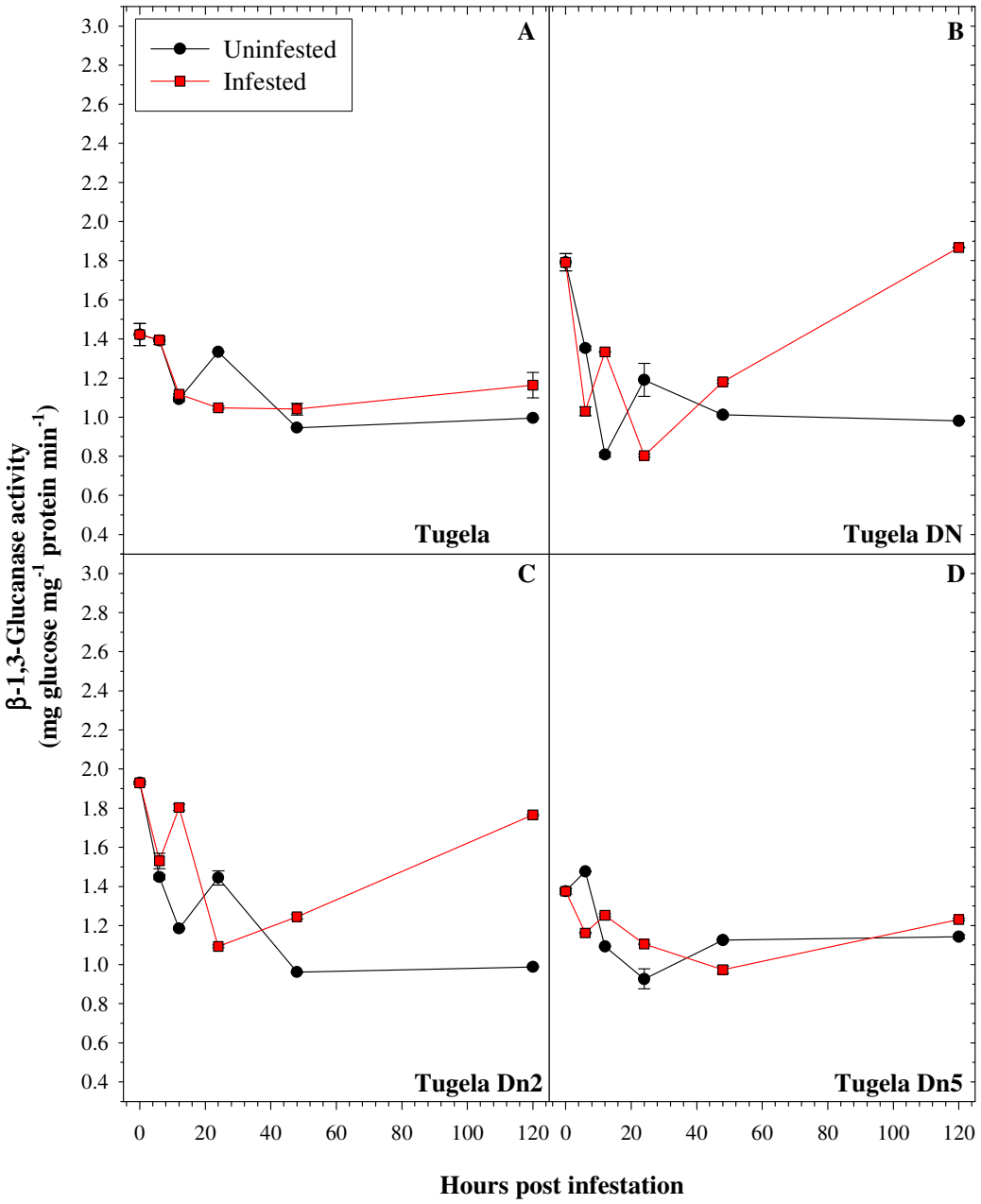


Fig. 4.1 Standard curve relating the absorbancies at 540 nm of the coloured product formed in the test for reducing sugars to glucose concentrations. (A) Depicts the linear region of (B) the complete curve.

I


Uninfested
Infested
A
B
C
D
Tugela
Tugela DN
Tugela Dn2
Tugela Dn5
β-1,3-Glucanase activity
(mg glucose mg-1 protein min-1)
Hours post infestation
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
0
20
40
60
80
100
120

II

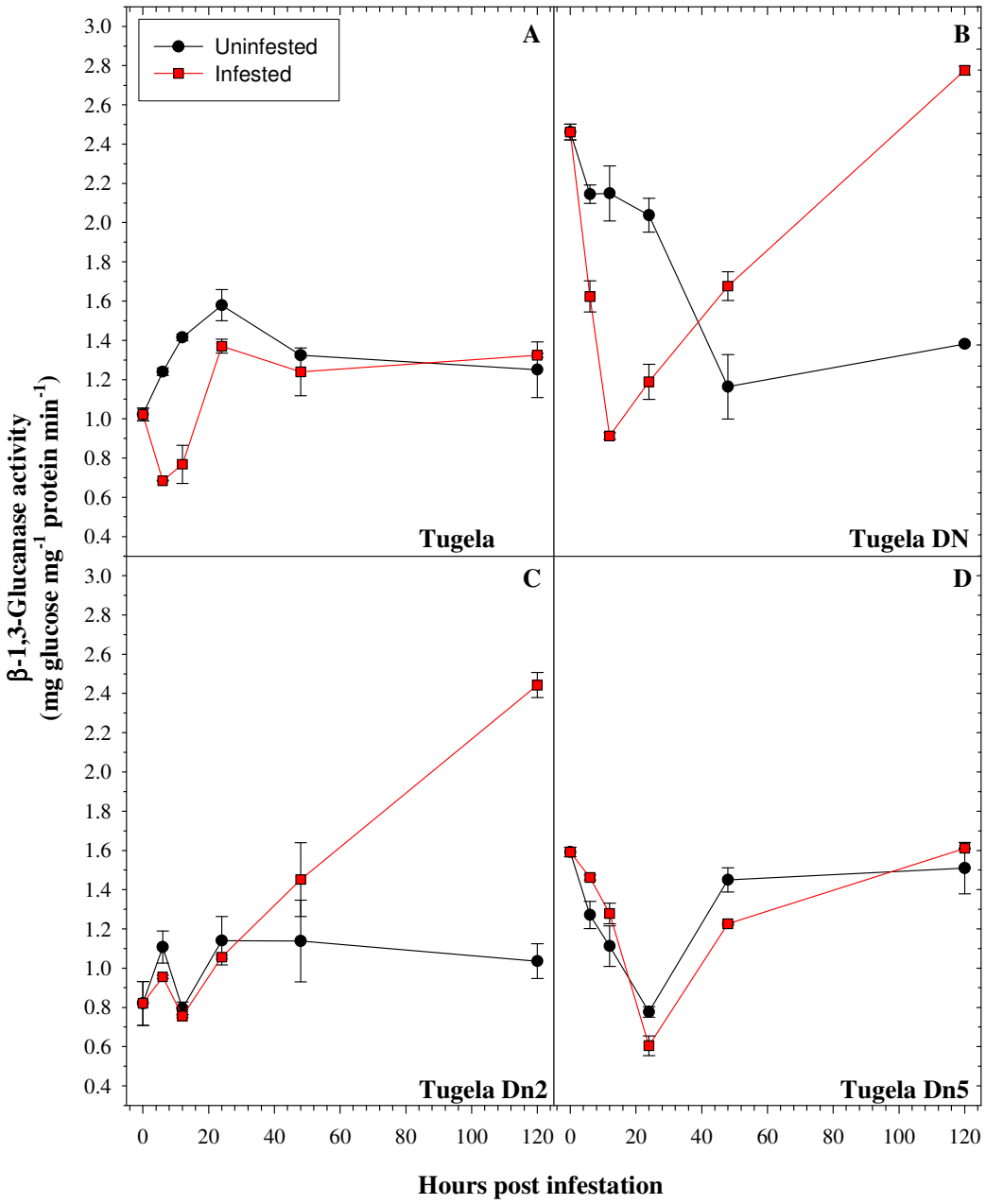


Fig. 4.2 Effect of RWA infestation on the intercellular β-1,3-glucanase activities of susceptible (cv. Tugela) (A) and resistant Tugela plants containing the Dn1 (B), Dn2 (C) and Dn5 (D) resistance genes respectively. I and II represent duplicate experiments. Error bars indicate standard deviation (n=3).

4.1.3. Apoplastic protein composition

Components of the resistance response should be reflected in a comparative study of protein compositions of uninfested and infested resistant and susceptible plants. Polypeptide profiles obtained by SDS-PAGE are images of the apoplast protein composition.

Visibly the polypeptide profiles of proteins from the uninfested susceptible plants were virtually similar to that of uninfested resistant plants during the investigation period (Fig. 4.3 and 4.4). The peptide levels remained relatively constant as the experiment proceeded (Fig. 4.3 and 4.4).

According to the polypeptide profiles, infestation did not affect the protein composition of susceptible plants qualitatively or quantitatively (Fig. 4.3 A). However, the polypeptide profiles of proteins from the infested resistant (cv. Tugela DN and Dn2) plants were different from that of uninfested plants (Fig. 4.3 B; 4.4 A and B). Two proteins, 31 and 14.96 kDa were induced in the infested resistant plants of cultivars Tugela DN and Dn2, but not in the uninfested plants (Fig. 4.3 B and 4.4 A). Infestation did not induce these proteins in the resistant (cv. Tugela Dn5) plants (Fig. 4.4 B).

4.1.4. Western blot analyses of β-1,3-glucanase expression

Western blots were performed to establish whether the higher β-1,3-glucanase activities found in the infested Dn1 and Dn2 plants, were the result of increased β-1,3-glucanase protein levels.

The β-1,3-glucanase antibody cross-reacted with two polypeptides (32.73 kDa and 33.11 kDa) in the uninfested susceptible plants (Fig. 4.5 A). The levels of the two peptides remained constant throughout the 120 h experimental period. The infested susceptible plants had the same size peptides cross-reacting with the β-1,3-glucanase antibodies. The levels of both these peptides increased slightly at 120 h of infestation (Fig 4.5 A).

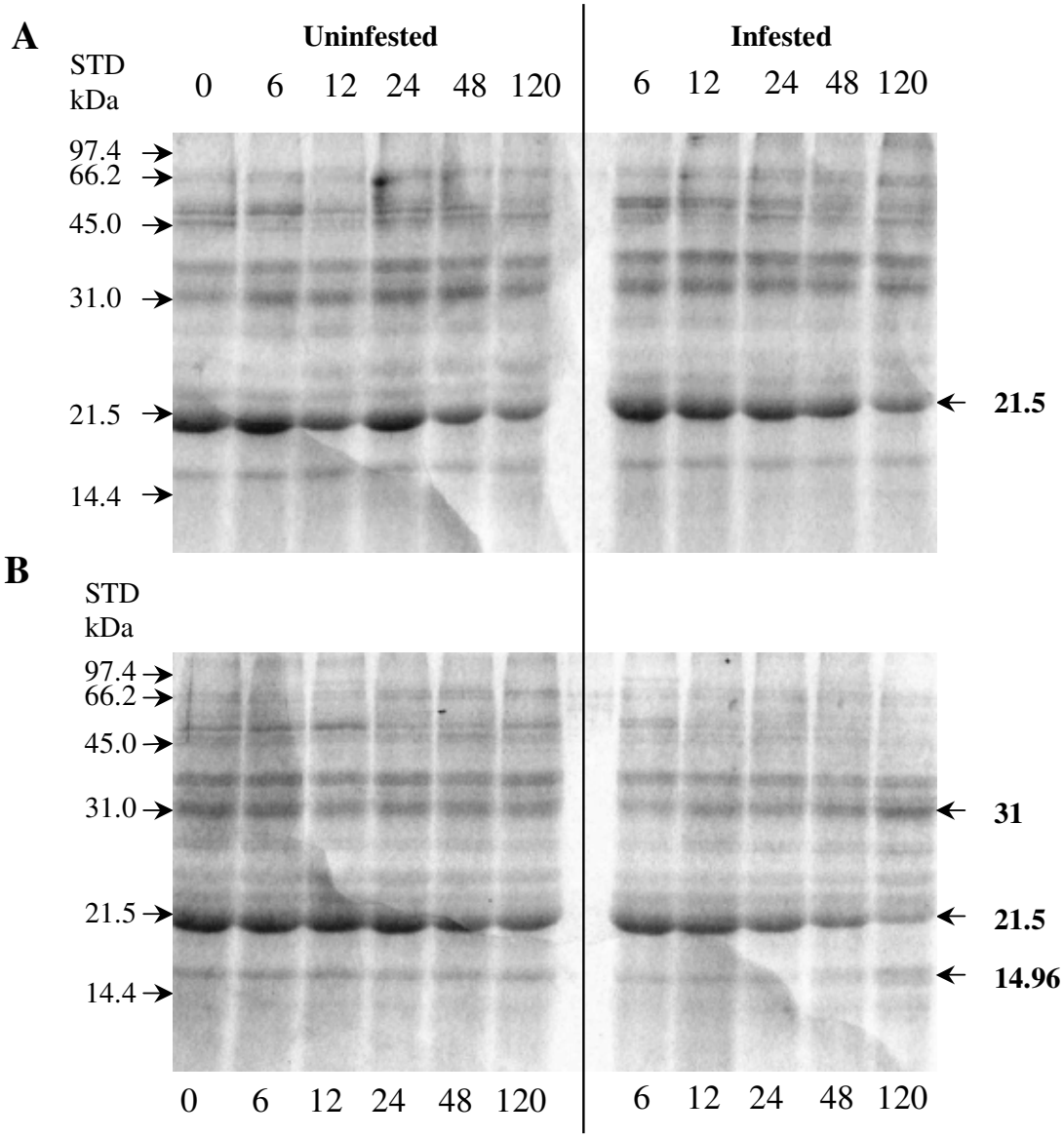


Fig. 4.3. SDS-PAGE polypeptide profiles of intercellular proteins from uninfested and infested susceptible cv. Tugela (A) and resistant cv. Tugela DN (B) plants.

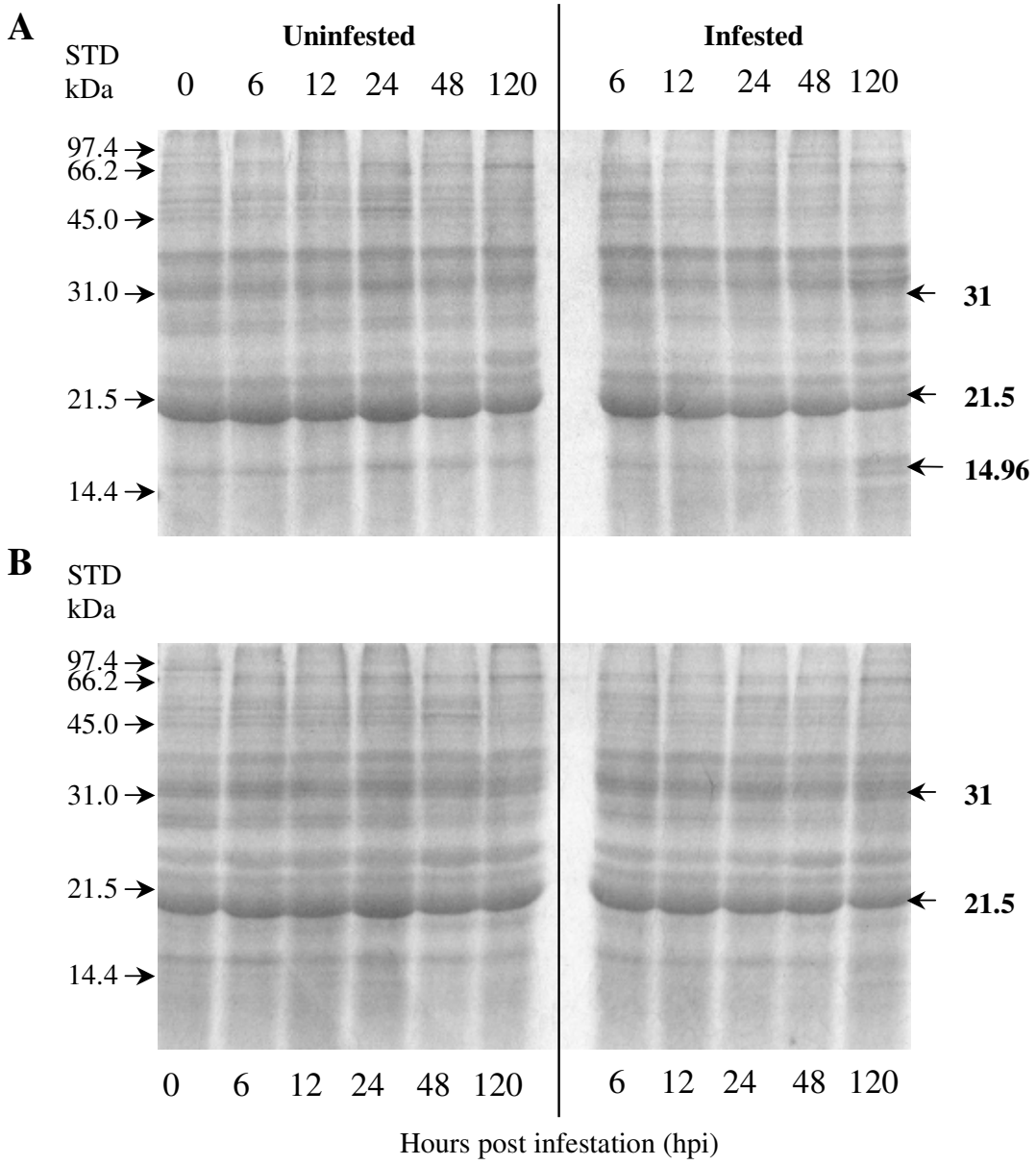


Fig. 4.4. SDS-PAGE polypeptide profiles of intercellular proteins from uninfested and infested resistant cv. Tugela Dn2 (A) and cv. Tugela Dn5 (B) plants.

In the resistant Tugela plants (cv. Tugela DN and Dn2), the β-1,3-glucanase antibodies cross-reacted with four polypeptides (31.62, 32.73, 33.11 and 34.28 kDa). In the uninfested plants, the protein levels remained constant throughout the 120 h experimental period. Infestation of the resistant plants substantially changed the expression of these peptides. Within 24 h, notable increases in the levels of all four polypeptides were found where after the levels further increased as the experiment proceeded to 120 hpi (Fig 4.5 B & C). Additionally, a unique 30.73 kDa polypeptide was present at 120 hpi in only the infested Tugela DN and Tugela Dn2 plants. This polypeptide did not occur in the infested Tugela and Tugela Dn5 plants.

Only three of the four peptides present in the Dn1 and Dn2 cultivars were detectable in the Tugela Dn5 uninfested plants (32.73, 33.11 and 34.28 kDa). The expression levels of these peptides remained constant throughout the 120 h experimental period. Western blots of proteins from infested Tugela Dn5 plants did not show the same notable increases in polypeptide band intensities as observed with Tugela DN and Tugela Dn2 plants (Fig. 4.5 D). At 120 hpi a minor increase in the polypeptide band intensities was however visible. Also, in Tugela Dn5 plants, the cross-reacting proteins seem to be constitutively expressed at higher levels.

The Western blots showed that there was an induction of expression of the β-1,3-glucanase peptides after RWA infestation. This induction was the highest in the Tugela DN and Tugela Dn2 resistant plants. While the susceptible and Tugela Dn5 resistant plants showed a delayed, but poor induction of β-1,3-glucanase proteins upon infestation.

4.1.5. Northern blots

Western blots indicated that induced β-1,3-glucanase activities coincided with increased β-1,3-glucanase protein levels. To establish whether the increase in protein content was also reflected on mRNA level and whether induction was controlled on transcriptionally, Northern blots were performed on the Tugela Dn1 and Tugela plants.

Both the barley and soybean β-1,3-glucanase probes recognized the same 1.2 kb mRNA (Fig. 4.6). Both probes resulted in similar mRNA expression profiles with signal intensities slightly lower for the soybean probe (Fig. 4.6 A and C). Uninfested susceptible plants (cv. Tugela) did not contain any detectable amounts of β-1,3-glucanase mRNA (Fig. 4.6 A and B). RWA

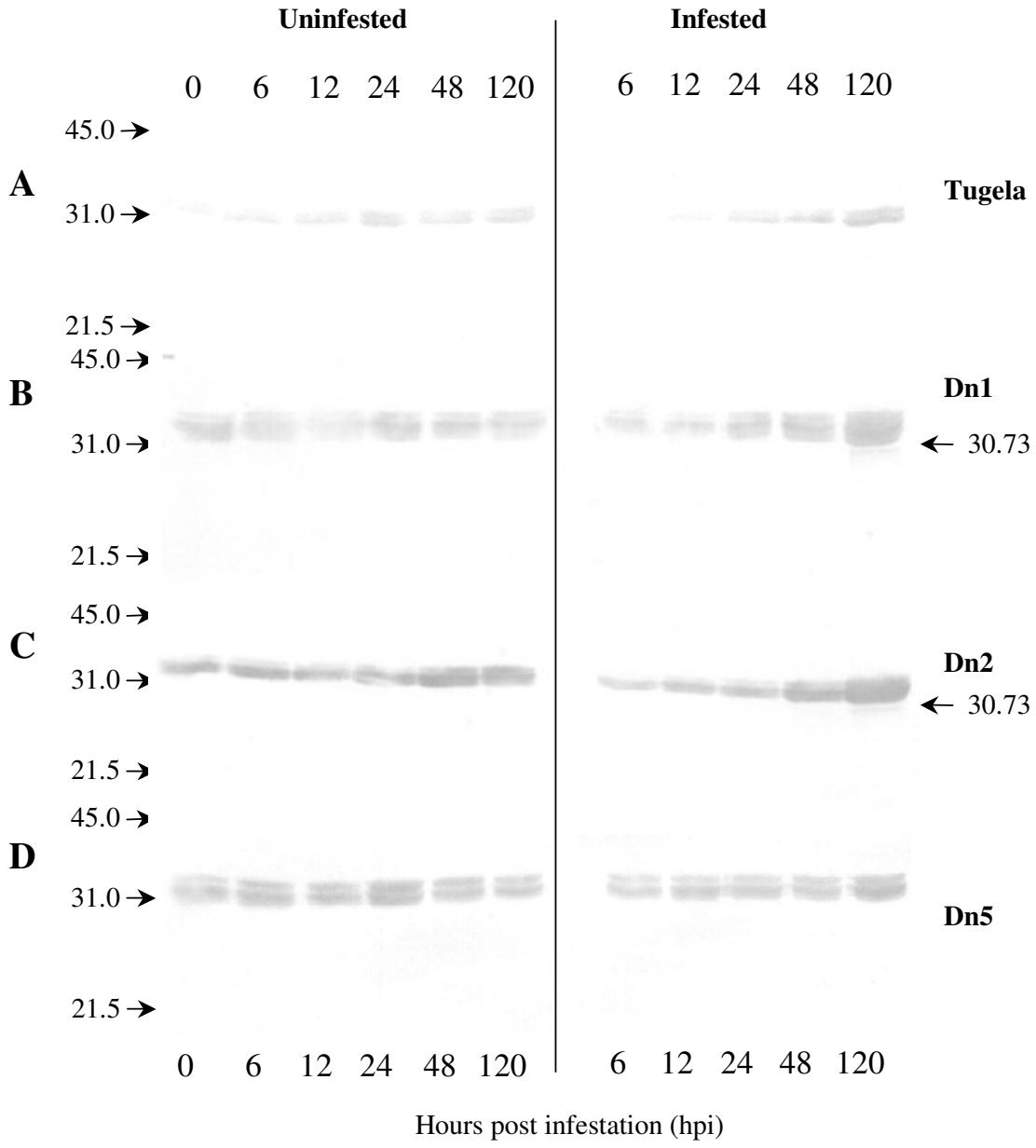


Fig. 4.5. Western blot analyses of intercellular β-1,3-glucanases from uninfested and infested susceptible Tugela (A), resistant Tugela DN (B), Dn2 (C) and Dn5 (D) plants. The molecular masses of the polypeptides are indicated in kDa.

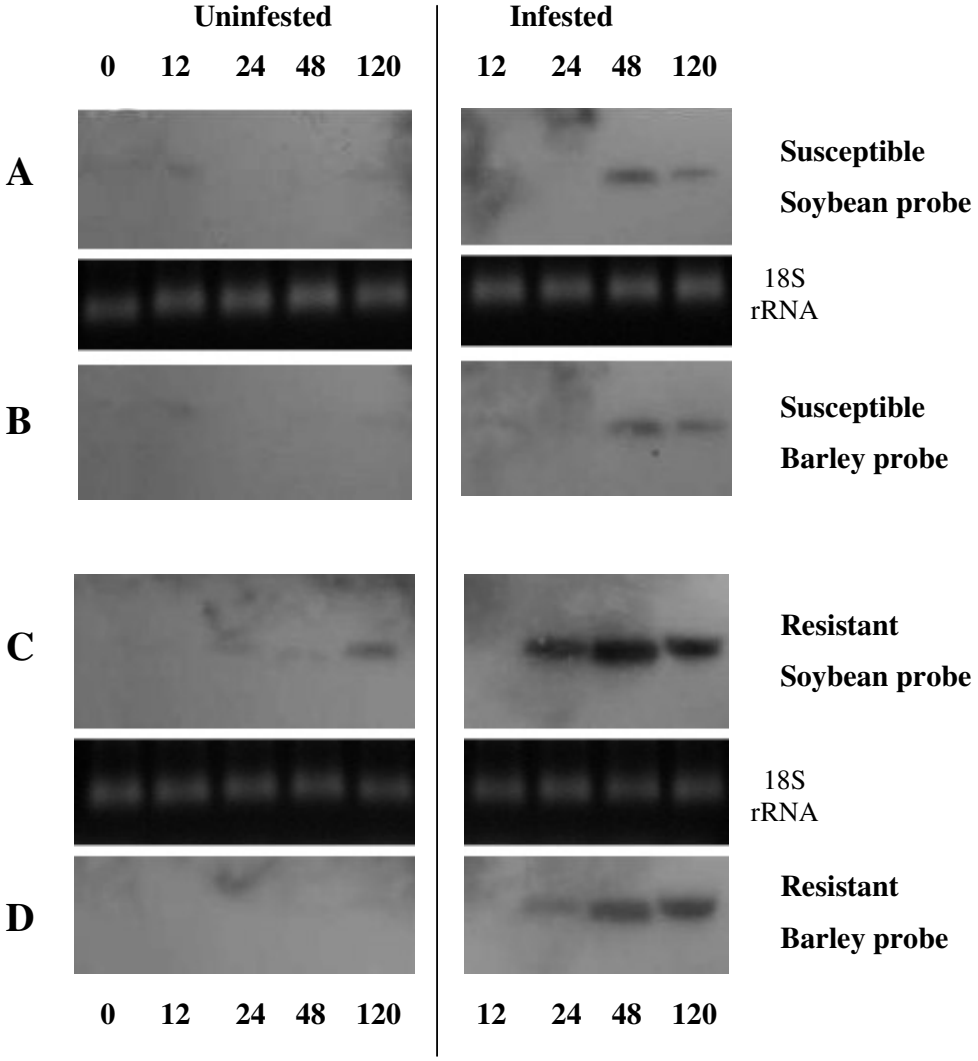


Fig. 4.6. Northern blot analyses of total RNA isolated from infested and uninfested susceptible (cv. Tugela) and resistant (cv. Tugela DN) plants. mRNA was probed with a soybean (A & C) and barley (B & D) β-1,3-glucanase DNA probe respectively.

infestation induced mRNA expression in susceptible plants 48 hpi. Slightly lower mRNA quantities seem to be present 120 hpi (Fig. 4.6 A and B).

In the uninfested resistant plants (cv. Tugela DN), very low levels of β-1,3-glucanase mRNA were detected 120 hpi (Fig. 4.6 C and D). RWA infestation of resistant (cv. Tugela DN) plants led to a substantial increase in mRNA levels 24 hpi. which reached a maximum at 48 hpi (Fig. 4.6 C and D) and then decreased.

4.2. IN SITU β-1,3-GLUCANASE LOCALISATION

4.2.1. Evaluation of the fixation procedure

The fixation of plant material used for *in situ* localisation is a critical step. The effect of osmium tetroxide and/or glutaraldehyde fixation on the ultrastructural preservation and epitope stability needed to be verified. Osmium tetroxide fixation has been shown to give false labelling while it also damages the epitopes of some proteins (Stirling, 1995). With this in mind, the effect of osmium tetroxide fixation on β-1,3-glucanase detection was investigated.

Superb resolution of the ultrastructure of leaf cells was obtained after osmium tetroxide-fixation (Fig. 4.7 A to C). In the cytoplasm, structures like the chloroplasts, mitochondria and in some cases the endoplasmic reticulum (ER) and Golgi apparatus could be distinguished from the surrounding cytoplasm (Fig. 4.7 A and B). Leaf tissues such as the vascular bundle and mesophyll cells were clearly visible (Fig. 4.7 C). Inside the vascular bundle, individual cells such as the phloem elements with their companion cells and the xylem elements containing substantial secondary thickenings, were easily distinguishable from the surrounding mesophyll cells, which make up the bulk of the leaves (Fig. 4.7 C).

When glutaraldehyde fixation was used on its own, it resulted in satisfactory ultrastructural preservation although not as good as in the case of osmium tetroxide fixation (Fig. 4.7 D to F). The membranes were not discernible as was the case with the osmium tetroxide staining. Other membrane-bound organelles such as the mitochondria and chloroplasts also seemed to lack their surrounding membranes (Fig. 4.7 D and F). However, inside these organelles, the fine organelle structures such as the grana in the chloroplasts and the cristae in the mitochondria were clearly distinguishable. These images were similar to a “negative image” compared to the osmium tetroxide stained samples where membranes were darker than the

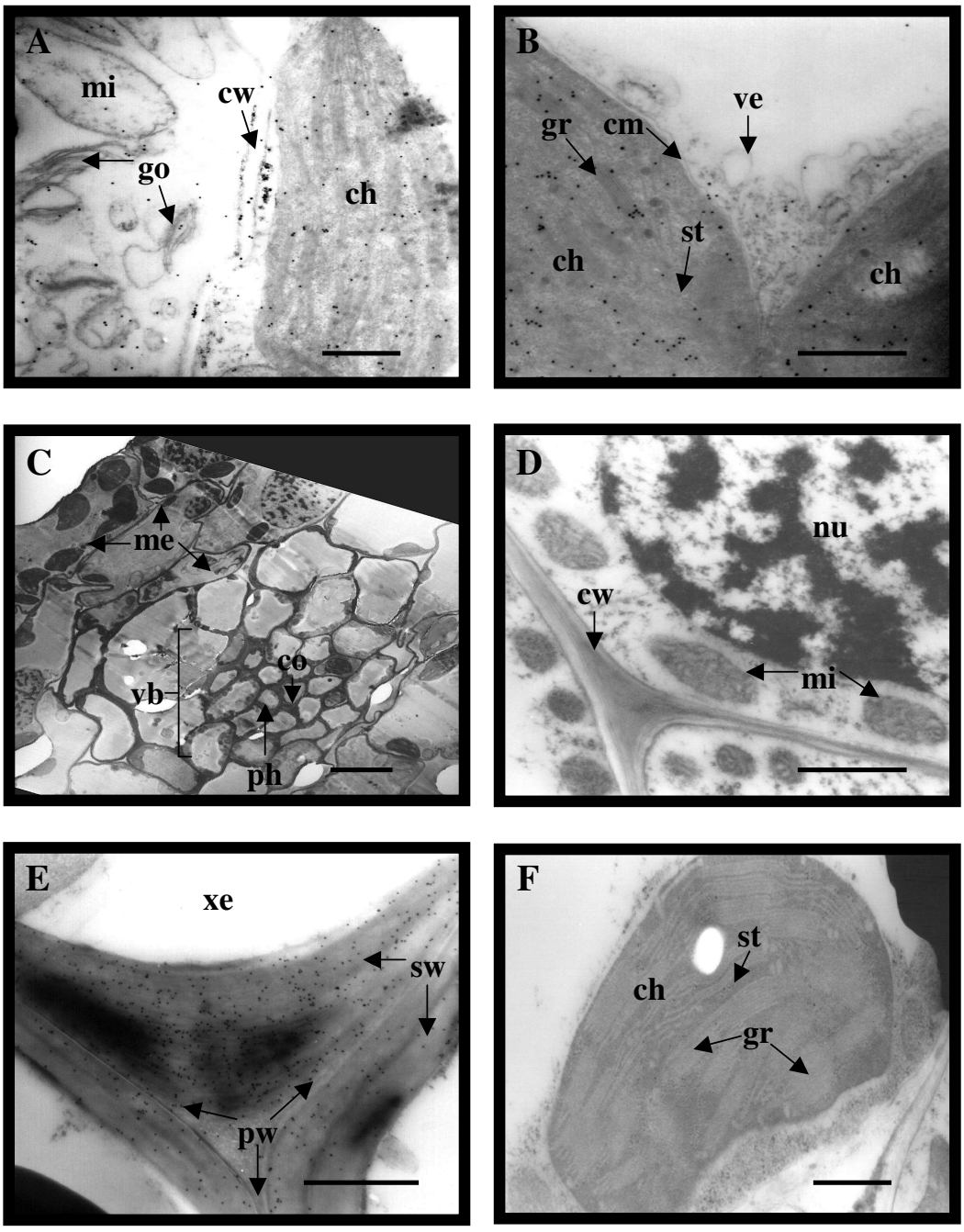


Fig. 4.7. Leaf sections treated with (A-C) and without osmium (D-F) showing (A) cytoplasmic membranes, (B) chloroplasts, (C) vascular bundle surrounded by mesophyll cells, (D) mitochondria and nucleus, (E) secondary thickenings in cell walls and (F) negative staining in chloroplasts. The abbreviations are (ch) chloroplast, (cm) chloroplast membrane, (co) companion cell, (cs) cytosol, (cw) cell wall, (go) golgi, (gr) grana, (me) mesophyll cells, (mi) mitochondria, (nu) nucleus, (ph) phloem, (pw) primary cell wall, (sp) stomatal pore, (st) stroma, (sw) secondary cell wall, (va) vacuole, (vb) vascular bundle, (ve) vesicle, (xe) xylem element.

surrounding tissue (Fig. 4.7 D and F). Other organelles such as the Golgi bodies, endoplasmic reticulum and secretory vesicles were, however, not discernible.

To evaluate the possible effect of osmium tetroxide fixation on the β-1,3-glucanase epitopes, a Western blot was performed with and without osmium tetroxide treatment. The untreated Western blot had detectable amounts of β-1,3-glucanases at all the dilutions (Fig. 4.8 A). However, after treatment with osmium tetroxide, the β-1,3-glucanase peptides present in the 10 µg protein sample could not be detected (Fig. 4.8 B). The osmium tetroxide therefore seemed to interfere with antibody binding to the epitope, which decreased the band intensity. For this reason, it was decided to perform the *in situ* localisation studies on plant material fixed only with glutaraldehyde.

In the wheat leaf sections, the β-1,3-glucanase antibodies cross-reacted with β-1,3-glucanases present in different tissues and cellular compartments (Fig. 4.7). The labelling occurred on the mesophyll and vascular bundle cell walls as well as on the chloroplasts (Fig 4.7 A, B and E). The method of fixation did not seem to have an effect on the distribution of the labelling. It is only when a comparison is made between the labelling densities that lower labelling densities on the osmium tetroxide fixed samples could be observed. Osmium tetroxide treated samples had much lower labelling densities compared to the sections fixed with glutaraldehyde (data not shown).

Only on the osmium tetroxide treated sections could the labelling that was present in the cytoplasm, be seen as localized in the Golgi apparatus and ER and not randomly distributed in the cytoplasm (Fig. 4.7 A).

4.2.2. Localisation and quantification of β-1,3-glucanases

The β-1,3-glucanase activity was determined to assess whether RWA infestation had induced β-1,3-glucanase expression in the material used for sectioning and localisation studies. The activity of uninfested plants of both resistant and susceptible plants remained low during the investigation period (Fig. 4.9A). In contrast, RWA infestation induced a substantial increase in activity in the resistant plants (Fig. 4.9 B). After 48 and 336 hpi the activity of infested plants were 34% and 80% higher compared to the uninfested plants (Fig. 4.9).

Labelling densities calculated from the images (Fig. 4.11 to 4.15) are indicated in Fig. 4.16 A, B and C.

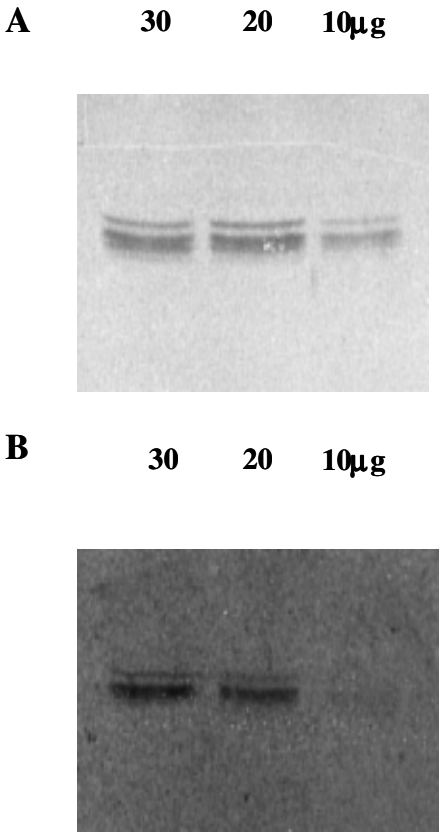


Fig. 4.8 Effect of osmium tetroxide treatment on the cross-reacting β-1,3-glucanase band intensities of a Western blot. (A) represents the untreated Western blot and (B) the osmium tetroxide treated Western blot. The amounts (µg) of protein loaded are indicated.

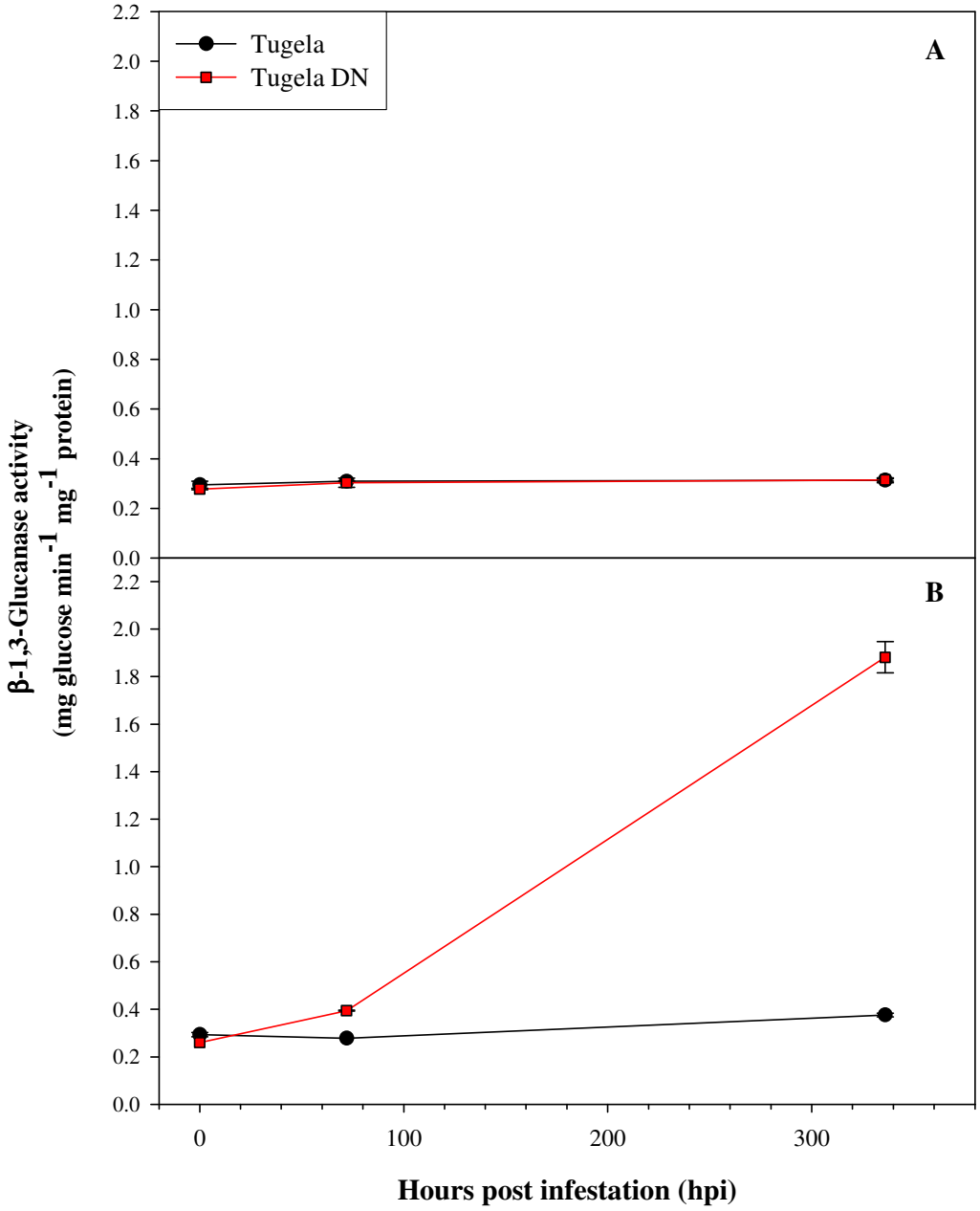


Fig. 4.9 β-1,3-glucanase activity in (A) uninfested and (B) infested sectioned wheat leaves. Error bars indicate standard deviation (n=3).

Pre-immune serum:

With the pre-immune serum little or no labelling occurred on the sections (Fig. 4.10 A to F). Some labelling could be observed on the cell walls of the xylem elements of susceptible (A) and resistant (B) plants (indicated with arrows). Insignificant labelling occurred on the chloroplasts and cell walls of mesophyll cells (Fig. 4.10 C to F). The little labelling which was present was randomly distributed over all the sections of resistant and susceptible plants (Fig. 4.10 A to F.

Uninfested plants at 0, 48 and 336 h:

At time zero, the β-1,3-glucanase antibodies cross-reacted with β-1,3-glucanases present in very low quantities in the uninfested susceptible and resistant leaf sections. In the susceptible plants very low labelling was found on the xylem cell walls (Fig. 4.11 A), chloroplasts (Fig. 4.11 C), mesophyll cell walls and cytoplasm (Fig. 4.11 E). The leaf sections of resistant plants at time zero showed similar distribution of labelling as susceptible plants, but labelling was much higher on the vascular bundle cell walls (Fig. 4.11 B and 4.16 A) and chloroplasts (Fig. 4.11 D and 4.16 C). The distribution pattern and labelling density in sections of uninfested susceptible and resistant plants at 48 h (Fig 4.12) and 336 h (Fig. 4.13) were similar to that at time zero.

Infested resistant vs. infested susceptible plants:

Infestation brought about a differentiation in labelling densities between resistant and susceptible plants. In uninfested susceptible plants, labelling was very low (Fig 4.12; 4.13 A, C and E) with no large increases in labelling densities after infestation (Fig. 4.14 A, C and E; Fig. 4.16 A; B and C). Labelling densities were higher in uninfested resistant plants (Fig 4.12; 4.13 B, D and F) than uninfested susceptible plants (Fig. 4.16). However after infestation significant increases in labelling occurred on the sections of resistant plants (Fig. 4.16 A, B and C).

Cellular component labelling:

Although labelling densities differed between susceptible and resistant plants, the labelling was restricted to the same cellular components. The highest levels of labelling were found in the vascular bundle cell walls (Fig. 4.16 A), mesophyll cell walls (Fig. 4.16 B) and the chloroplasts of the mesophyll cells (Fig. 4.16 C). Cytoplasm labelling was not

significantly different between different plant sections and remained very low and was therefore not included. The highest labelling densities were observed in the chloroplasts (Fig. 4.15 D and F; 4.16 C) of infested resistant plants. Higher labelling densities were found in the cell walls of vascular bundles of uninfested resistant than susceptible plants. Infestation further increased labelling densities in the vascular bundle cell walls of resistant plants (Fig. 4.16 A).

Increases in labelling over investigation period:

As the infestation time progressed, increases in labelling could be observed in the different cellular components. Notable increases were present after 48 h of infestation in all the cellular components of resistant plants (Fig. 4.16 A, B and C). The labelling densities in the chloroplasts and mesophyll cell walls 336 h after infestation were almost 100% higher than 48 h after infestation of resistant plants (Fig. 4.16 B and C). This large increase in labelling did not occur in the infested susceptible plants in either the vascular bundle cell wall or mesophyll cell walls. The increase in chloroplast labelling of infested susceptible plants was delayed and could be observed 336 h after infestation (Fig. 4.16 C).

To review:

Labelling densities of uninfested and infested resistant plant sections were completely different. Labelling of uninfested resistant plants was low and remained relatively unchanged while in the infested resistant plants large increases in labelling occurred as the experiment proceeded. Labelling of infested and uninfested susceptible plant sections were very similar and only slightly increased labelling was found in the infested susceptible plants.

Labelling occurred on the same cellular components in both infested and uninfested susceptible and resistant plants. Intercellular labelling displayed large increases in labelling in both vascular bundle and mesophyll cell walls of infested resistant plants. Intracellular labelling was mostly restricted to chloroplasts and low quantities occurred in the cytoplasm, which was unaffected by infestation. Infestation of resistant plants resulted in high labelling of chloroplasts.

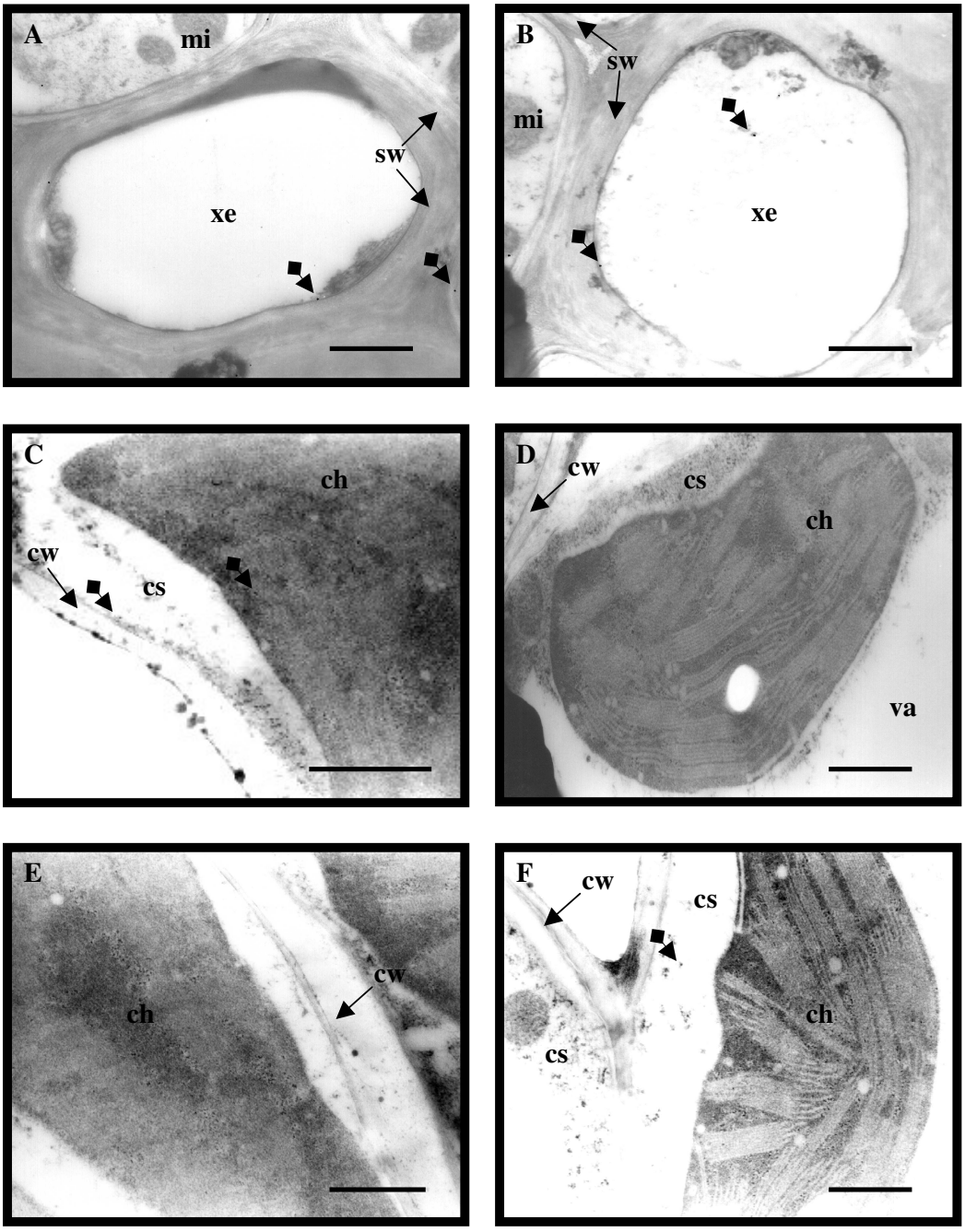


Fig. 4.10 Immunogold localisation of β-1,3-glucanase in infested susceptible (A, C and E) and resistant (B, D and F) plants at 336 hpi probed with pre-immune serum. A-F represents different sections with different cellular components. Abbreviations: (ch), chloroplasts; (cs), cytosol; (cw), cell wall; (mi), mitochondria; (sw), secondary cell wall; (va), vacuole; (xe), xylem element.

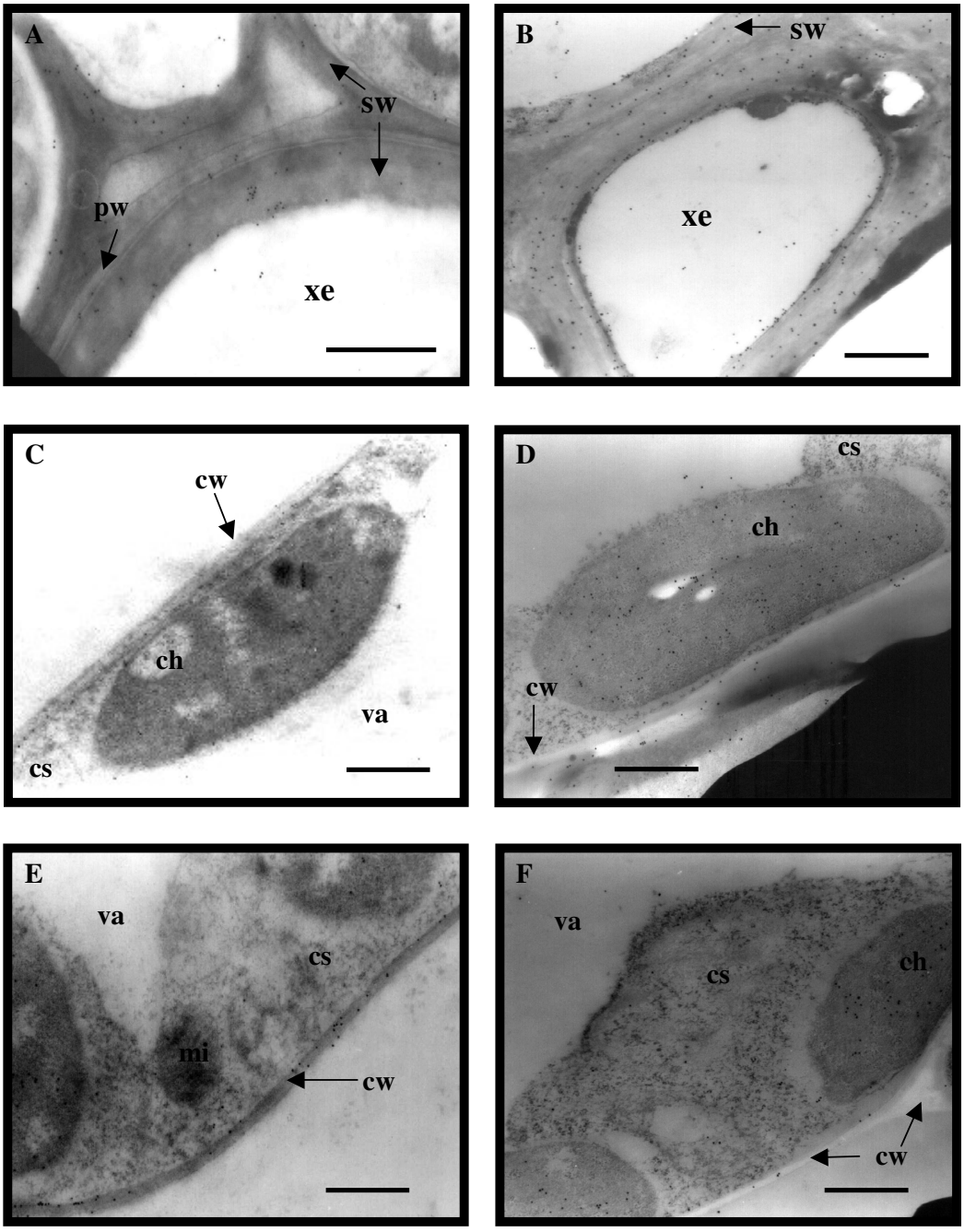


Fig. 4.11 Immunogold localisation of β-1,3-glucanase in susceptible (A, C and E) and resistant (B, D and F) plants at 0 hpi. A-F represents different sections with different cellular components. Abbreviations: (ch), chloroplasts; (cs), cytosol; (cw), cell wall; (mi), mitochondria; (pw), primary cell wall; (sw), secondary cell wall; (va), vacuole; (xe), xylem element.

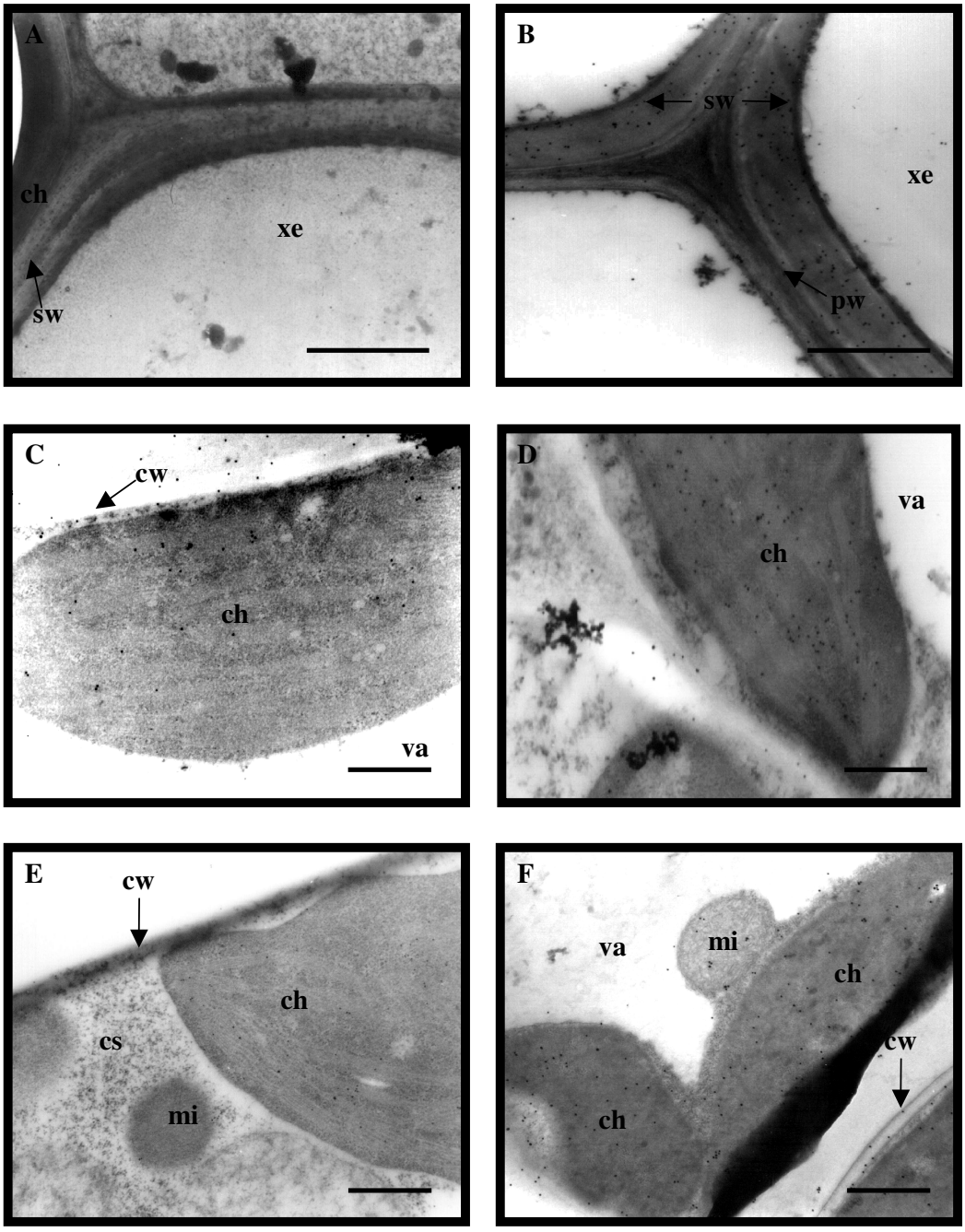


Fig. 4.12 Immunogold localisation of β-1,3-glucanase in uninfested susceptible (A, C and E) and resistant (B, D and F) plants at 48 hpi. A-F represents different sections with different cellular components. Abbreviations: (ch), chloroplasts; (cs), cytosol; (cw), cell wall; (mi), mitochondria; (pw), primary cell wall; (sw), secondary cell wall; (va), vacuole; (xe), xylem element.

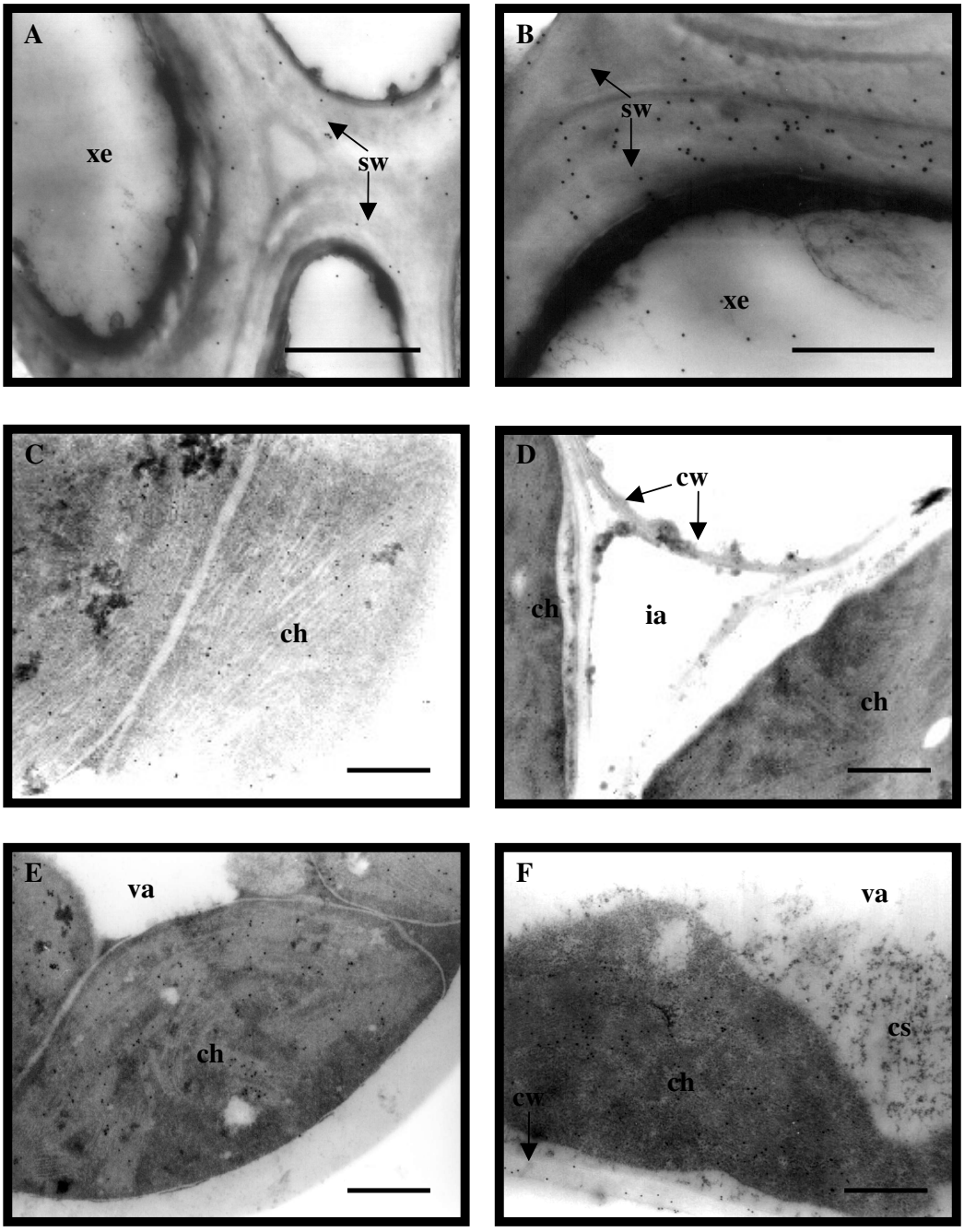


Fig. 4.13 Immunogold localisation of β-1,3-glucanase in uninfested susceptible (A, C and E) and resistant (B, D and F) plants at 336 hpi. A-F represents different sections with different cellular components. Abbreviations: (ch), chloroplasts; (cs), cytosol; (cw), cell wall; (mi), mitochondria; (pw), primary cell wall; (sw), secondary cell wall; (va), vacuole; (xe), xylem element.

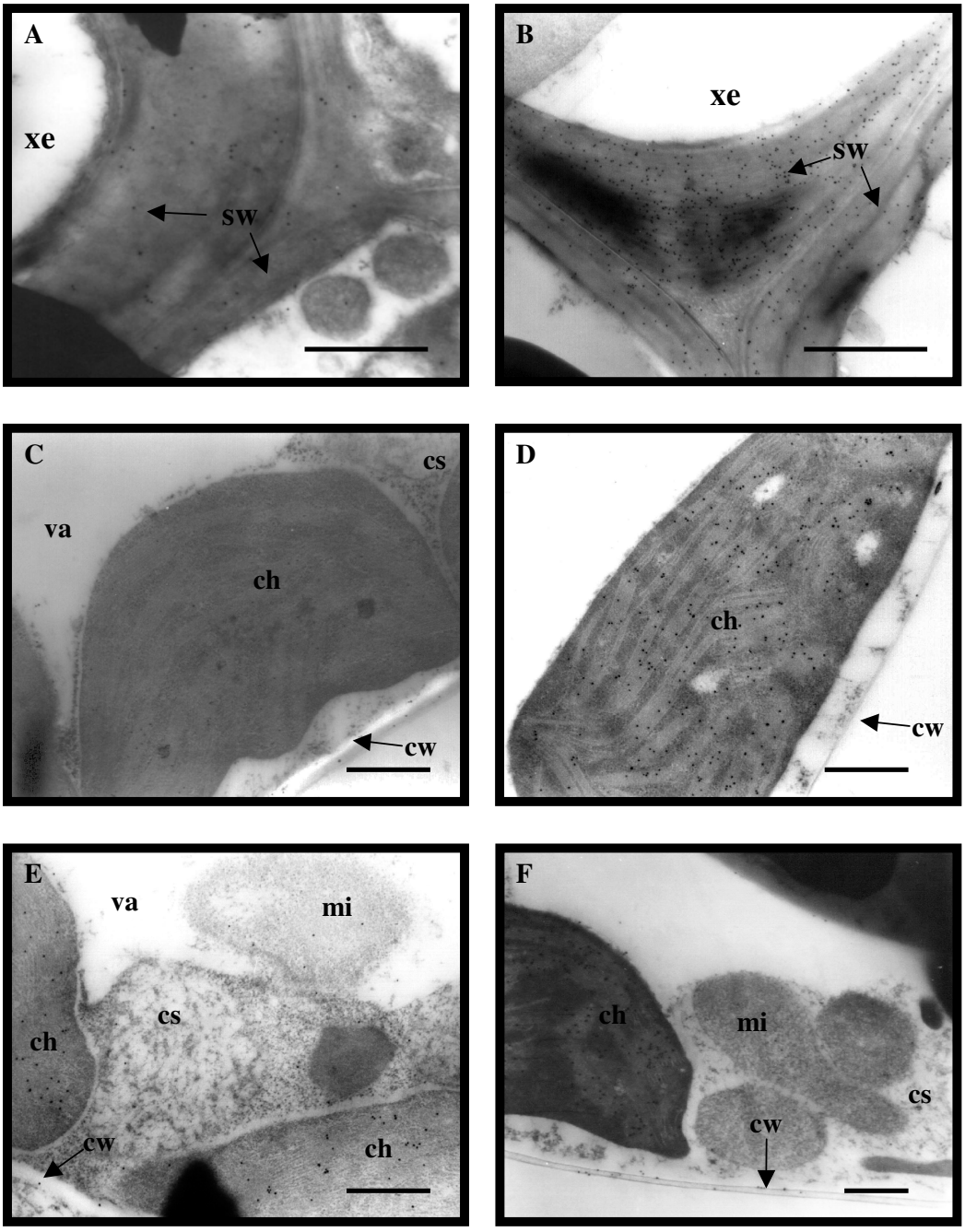


Fig. 4.14 Immunogold localisation of β-1,3-glucanase in infested susceptible (A, C and E) and resistant (B, D and F) plants at 48 hpi. A-F represents different sections with different cellular components. Abbreviations: (ch), chloroplasts; (cs), cytosol; (cw), cell wall; (mi), mitochondria; (pw), primary cell wall; (sw), secondary cell wall; (va), vacuole; (xe), xylem element.

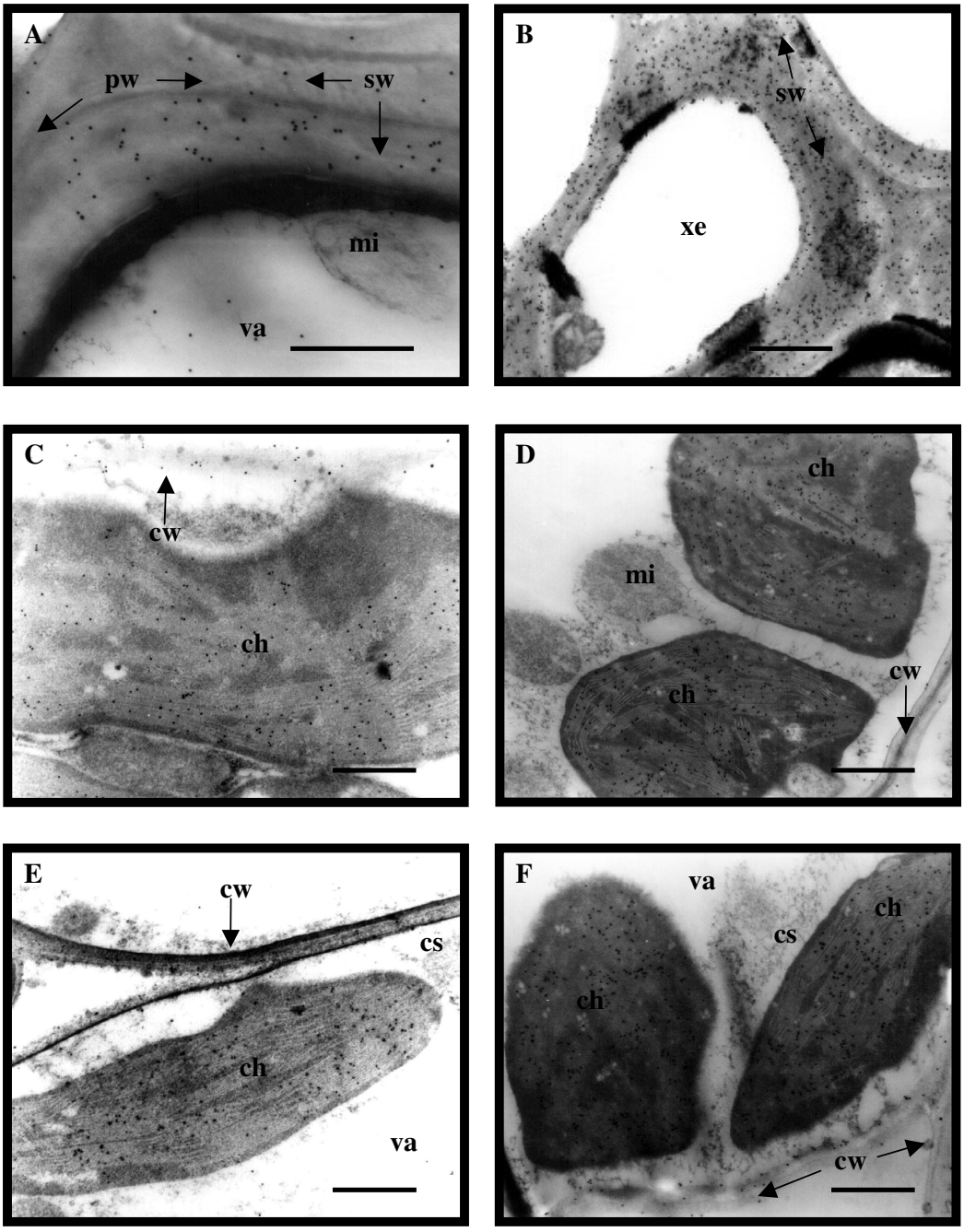


Fig. 4.15 Immunogold localisation of β-1,3-glucanase in infested susceptible (A, C and E) and resistant (B, D and F) plants at 336 hpi. A-F represents different sections with different cellular components. Abbreviations: (ch), chloroplasts; (cs), cytosol; (cw), cell wall; (mi), mitochondria; (pw), primary cell wall; (sw), secondary cell wall; (va), vacuole; (xe), xylem element.

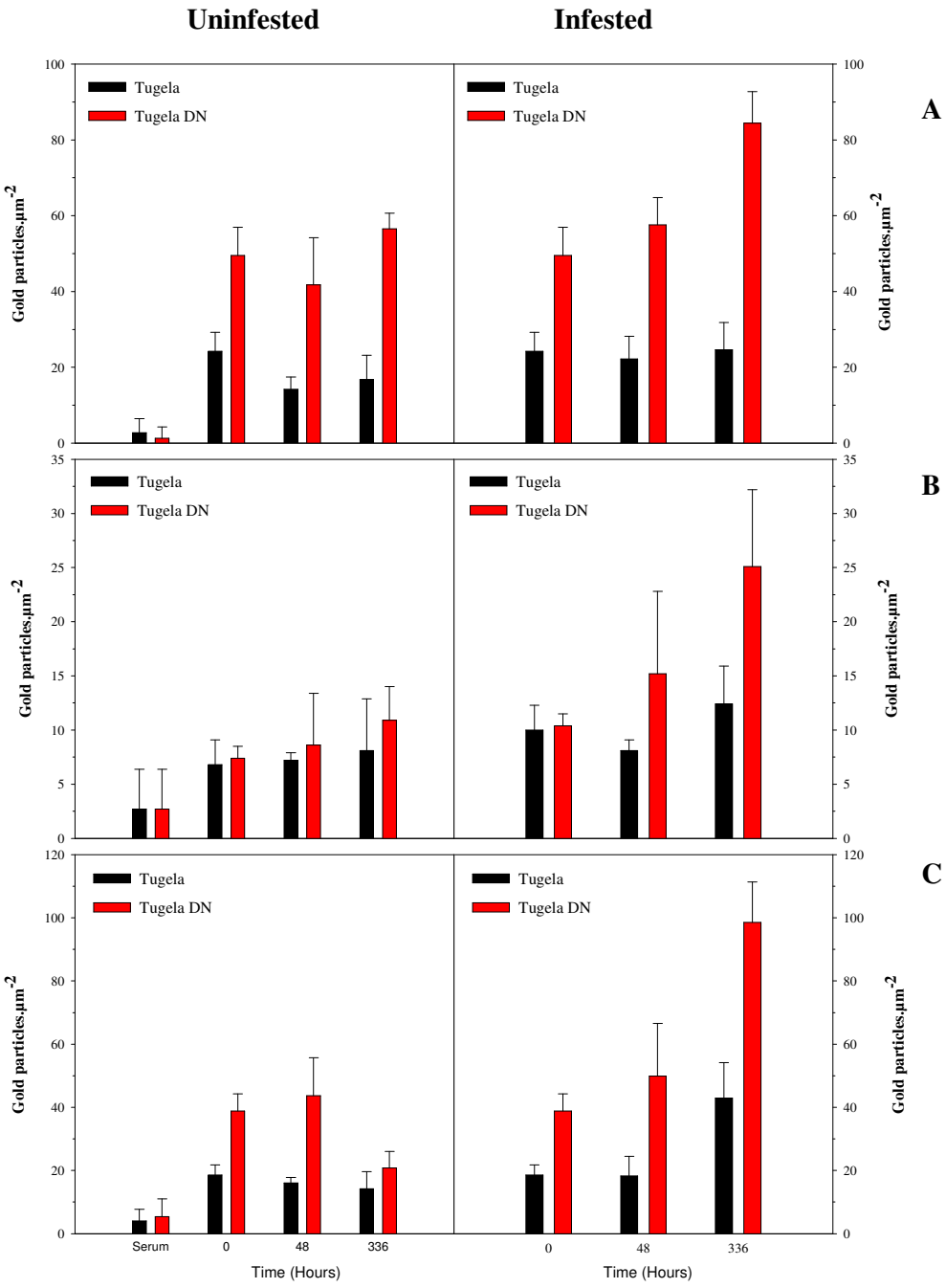


Fig. 4.16 Labelling density in the vascular bundle cell walls (A), mesophyll cell walls (B) and chloroplasts of mesophyll cells (C). Error bars indicate standard deviation (n=30).

4.3. β-1,3-GLUCANASES IN CHLOROPLASTS

Since the presence of β-1,3-glucanases in the chloroplasts as indicated in the localisation studies (e.g. Fig. 4.15 D) was novel, these results needed to be verified. For this reason chloroplasts were isolated and different chloroplast fractions tested for β-1,3-glucanase activity and the presence of β-1,3-glucanase cross-reacting proteins (Fig. 3.1).

4.3.1 β-1,3-glucanase activity in chloroplast fractions

The first fraction consisting of the total leaf extract (excluding the chloroplasts) contained high β-1,3-glucanase activity (Fig. 4.17). Infestation differentially induced the β-1,3-glucanase activity of this fraction to much higher levels in the resistant plants than the susceptible plants.

Fraction 2 contained the unbound stroma proteins of the chloroplasts. The β-1,3-glucanase activity was comparatively low in this fraction and was relatively unaffected by infestation in both resistant and susceptible plants (Fig. 4.17).

High levels of β-1,3-glucanase activity resided in the chloroplast membrane bound fraction of infested resistant plants (Fig. 4.17 B). Only about a third of the β-1,3-glucanase activity was present in the infested susceptible plants compared to the infested resistant plants membrane bound fraction(Fig. 4.17 A). The uninfested susceptible and resistant had much lower β-1,3-glucanase activities (Fig. 4.17 A).

4.3.2. Western blot analyses of chloroplast proteins

To assess the validity of the β-1,3-glucanase activity in the chloroplasts, Western blots were performed on each chloroplast fractionation.

The β-1,3-glucanase antibodies cross-reacted with two proteins present in the total leaf fraction of the susceptible plants (32.7 and 33 kDa) and three in the resistant plants (32.7, 33.1 and 34 kDa) (Fig 4.18). No β-1,3-glucanase proteins were detected in the unbound stroma proteins.

A 35 kDa β-1,3-glucanase protein occurred in the chloroplast membrane bound fraction. The infested resistant plants contained higher concentrations of this β-1,3-glucanase isoform (Fig.

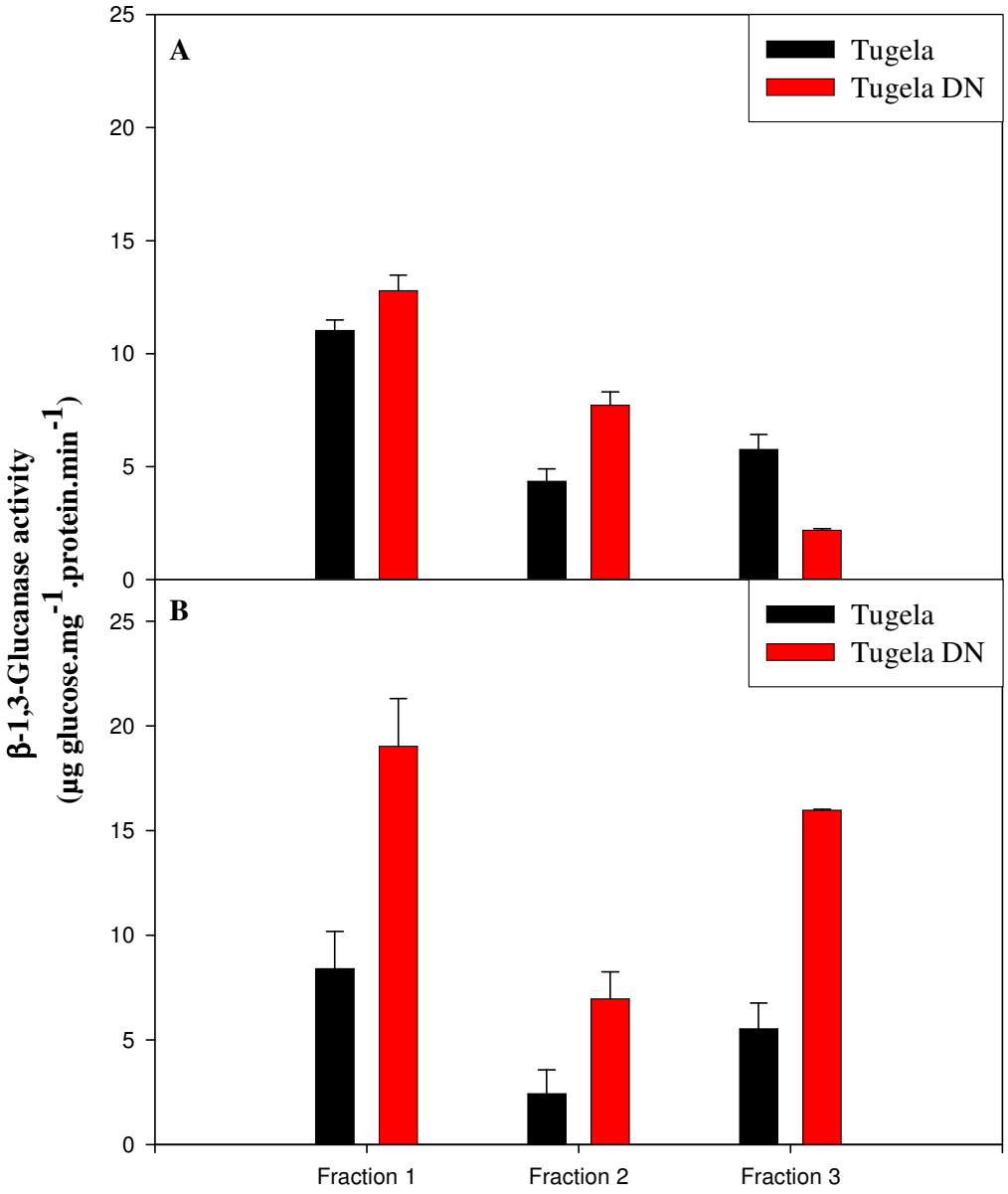


Fig 4.17 β-1,3-glucanase activities in different chloroplast fractions of uninfested (A) and infested (B) resistant (cv Tugela DN) and susceptible (cv Tugela) plants. Fraction 1 is the total leaf extract, fraction 2 is the chloroplast stroma and fraction 3 is the chloroplast membrane bound fraction (Fig. 3.1).

4.18 D) compared to the uninfested resistant (Fig. 4.18 C), and uninfested (Fig. 4.18 A) and infested susceptible (Fig. 4.18 B) plants. This indicates the possibility that some membrane-bound β-1,3-glucanase do reside within the chloroplast membranes.

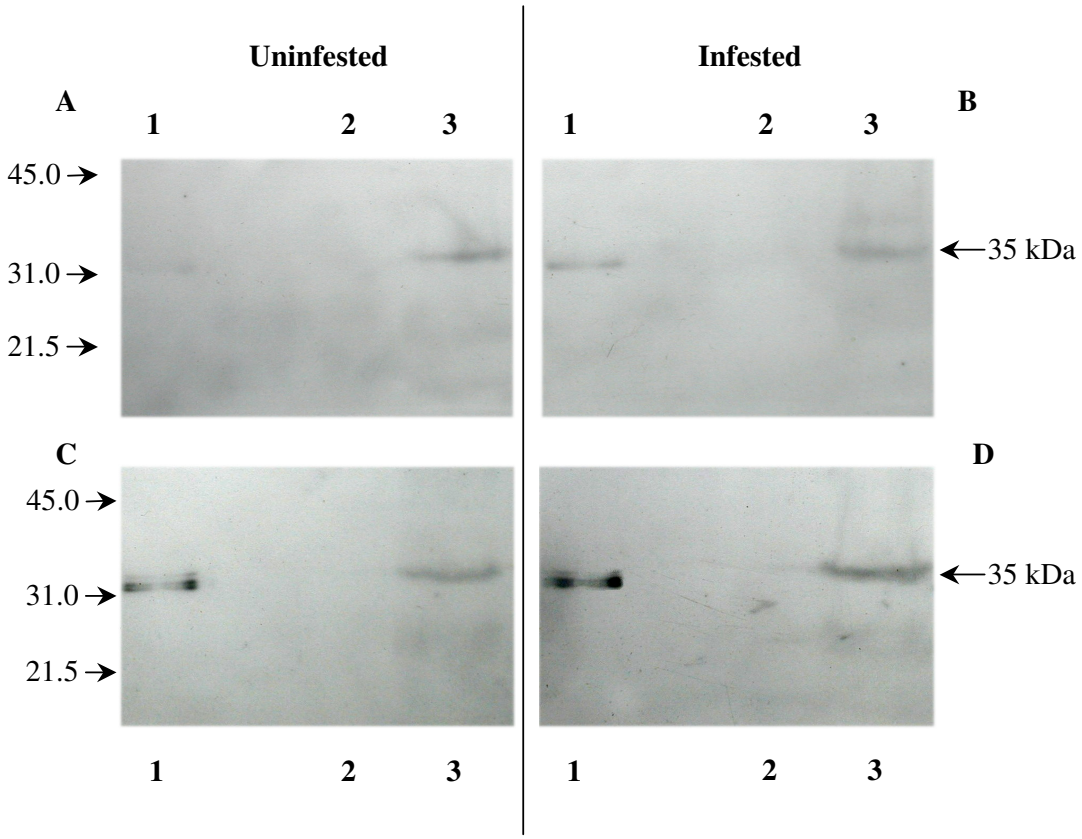


Fig. 4.18 Western blot analysis of β-1,3-glucanases in the different chloroplast fractions. Lane 1 is the total leaf extract, lane 2 the chloroplast stroma and lane 3 the chloroplast membrane fraction (Fig. 3.1) of uninfested susceptible (A), infested susceptible (B), uninfested resistant (C) and infested resistant (D) plants. Arrows indicate the molecular sizes (kDa).

CHAPTER 5

DISCUSSION

The hypersensitive cell death is a common resistance mechanism deployed against microbial pathogens and the synthesis of PR proteins is one of the characteristic biochemical changes. Two of the PR proteins, β-1,3-glucanase and chitinase, are plant hydrolases implicated in this defence against pathogens (Stintzi *et al*., 1993b). Fewer studies have been done on the HR associated with plant-insect interactions compared to pathogens. However, investigations showed that PR proteins are expressed as part of the resistance mechanisms against insects. Forslund *et al*., (2000) showed that barley plants resistant to birdcherry-oat aphid expressed β-1,3-glucanases at much higher levels than the susceptible plants upon infestation. Broderick *et al*., (1997) also found the induction of β-1,3-glucanases in clover (*Trifolium subtereneum*) with Redlegged earth mite attack.

β-1,3-glucanase is either directly detrimental to the pathogen or indirectly by releasing elicitors that activate defence genes (Bowles, 1990). The role of β-1,3-glucanases against aphids is however unclear (Forslund *et al*., 2000), but it must play a role since it is differentially expressed in the resistant plants after aphid infestation (Forslund *et al*., 2000). The expression of β-1,3-glucanases in Tugela wheat plants with different resistance genes (Dn1, Dn2 and Dn5) could help to give a clearer picture of its role in the defence mechanism. This is especially true since these different resistance genes have different modes of resistance (Smith *et al*., 1992).

The different resistance genes did affect the expression of β-1,3-glucanases upon RWA infestation. In Tugela DN and Tugela Dn2 plants, RWA infestation induced a substantial increase in β-1,3-glucanase activity (Fig. 4.2 IB and C; Fig 4.2 IIB and C). This large induction did not occur in infested resistant Tugela Dn5 plants or the susceptible plants (Fig. 4.2 IA and D; Fig. 4.2 IIA and D). Western blot analyses showed that the Dn5 resistant cultivar had only three of the five β-1,3-glucanase peptides present in the other two resistant cultivars (Fig. 4.5 D).

Other studies have shown that plants with the Dn5 resistance gene had smaller necrotic lesions compared to plants with Dn1 and Dn2 genes (Du Toit, 1988). Beffa *et al*., (1996) reported markedly reduced lesion size in transformed tobacco plants expressing an antisense copy of a β-1,3-glucanase gene. These tobacco plants had lower β-1,3-glucanase activity, which led to higher callose deposits around and in necrotic lesions caused by viral infections.

The smaller necrotic lesions of infested Dn5 plants could possibly be related to lower β-1,3-glucanase expression. Localisation of accumulated callose in the Dn5 plants when compared to Dn1 and/or Dn2 plants would indicate whether differences exist between these plants.

Large increases in β-1,3-glucanase proteins were detected in the infested resistant Tugela DN and Dn2 plants (Fig. 4.5 B and C). In both of these resistant lines, up to five individual isoforms were detected with a unique 30.73 kDa peptide detectable at 120 h. Van der Westhuizen *et al*., (1998a) using IEF activity gels, detected 7 intercellular β-1,3-glucanase isoenzymes in wheat infested with RWA. The isoenzymes were selectively induced to higher levels in the resistant than in the susceptible cultivar. When equal activities were loaded, all the isoenzymes were present in uninfested and infested resistant and susceptible Tugela plants. It is, therefore expected that the "unique" peptide detected in the Dn1 and Dn2 resistant cultivars are present at non-detectable levels in the susceptible and Dn5 plants. Van der Westhuizen *et al*., (1998a) concluded that no specific isoenzymes were linked with resistance or susceptibility. However, the level or localisation of the expressed isoenzymes is expected to be important in the resistance response.

In the Northern blots, a single major transcript hybridised with both DNA probes (Fig. 4.6). Roulin *et al*., (1997) showed that the two main β-1,3-glucanase isoforms accumulating in barley after infection with leaf scald fungus (*Rhynchosporium secalis*) were GI and GII, with the latter being the predominant isoform. GII was shown to have an extracellular targeting signal peptide and was mainly found in the IWF. The recognition by the GII DNA probe of β-1,3-glucanase mRNA induced by the RWA infestation (Fig. 4.6) indicates that the induced β-1,3-glucanases have high homology with the GII isoforms found in barley. Furthermore, GI and GII DNA probes were shown to detect only specific β-1,3-glucanase protein isoform mRNAs (Roulin *et al*., 1997). The soybean β-1,3-glucanase gene was isolated from an expression library synthesized from mRNA of ethylene treated soybean seedlings. It codes for a 33.5 kDa mature protein with a 2.5 kDa signal peptide responsible for targeting it to the intercellular space. It hybridised to a single size class of mRNA which showed a large induction after treatment with ethylene (Takeuchi *et al*., 1990). Even though high homology is not expected between soybean and wheat β-1,3-glucanases, this probe hybridised to the same size mRNA forming a single band as with the barley probe (Fig. 4.6 A and C). This could either mean both these probes hybridised to mRNA of the same isoenzyme or to different

isoenzymes with similar size mRNA which is highly likely considering the similar sizes of the polypeptides (30-34 kDa).

While the Western blot analyses confirmed that the increase in activity was due to increased protein levels, Northern blots suggested increased transcription of β-1,3-glucanase genes led to this increase. Another important difference between susceptible and resistant plants was apparent here. The susceptible plants had a delayed increase of mRNA only at 48 hpi (Fig. 4.6 A and B) while the Tugela DN plants already accumulated a large quantity of β-1,3-glucanase mRNA at 24 hpi (Fig. C and D). Roulin *et al*., (1997) using near-isogenic resistant and susceptible lines of barley, described an accelerated β-1,3-glucanase mRNA accumulation after scald fungus infection. A near full-length cDNA coding for isoenzyme GII indicated that mRNA of β-1,3-glucanases accumulated 24 h earlier in the resistant line compared to the susceptible line. In resistant melon cultivar, an increase in the β-1,3-glucanase mRNA transcripts was apparent 12 h after inoculation with *Sphaerotheca fusca*, while susceptible plants had barely detectable amounts after three days (Rivera *et al*., 2002). It would therefore seem that the induction time for increased gene expression of β-1,3-glucanase is an indication of the resistance mechanism in plants.

These results prove the early and rapid accumulation of β-1,3-glucanases in the RWA resistant plants after infestation. However, its role in the defence against RWA is unclear. Wubben *et al*., (1993) stated that the subcellular localisation of the induced β-1,3-glucanase enzymes *in planta* could provide important information about the role of this enzyme in the resistance mechanism against the invading pathogen. Immunogold localisation of the expressed β-1,3-glucanases could give valuable information about the possible role of β-1,3-glucanases in the defence against RWA.

The localisation of β-1,3-glucanases after insect or aphid infestation has not been studied very intensively. Lisbeth Jonsson (Sodertorn University College, Sweden) found that PR proteins expressed in barley after birdcherry-oat and rose-grain aphid infestation were restricted to the vicinity of aphid probing and feeding (personal communication). The most data thus presented relate to the location of the β-1,3-glucanases after different pathogen infections (Benhamou *et al*., 1989; Hu and Riijkenberg, 1998; Tenberge *et al*., 1999). Cold stress and ethylene treatment, which induce β-1,3-glucanases, have also been investigated (Mauch and Staehelin, 1989; Pihakaski-Maunsbach *et al*., 1996). These studies have shown that β-1,3-

glucanases can be expressed in different cellular compartments depending on the stimuli. It would be of great importance to investigate whether the same localisation of the expressed β-1,3-glucanases with pathogen infection is found in plants after RWA infestation.

Immunogold labelling (IGL) of ultrathin sections is a powerful technique for the detection of antigens in samples of embedded tissues. This technique depends on the exposure of antigen/epitope sites on the surface of the section, which are then recognized by antibodies, which in turn are recognized by a secondary antibody coupled to a colloidal gold particle (GAR). The recognition of a particular antigen depends on how many are present at the surface of the resin and therefore available for labelling (Van Lent *et al*., 1990). However, not all antigen sites on the surface are antigenic (Glasbey and Roberts, 1997).

The lower band intensity on the Western blot treated with osmium tetroxide is a clear indication of the damage the fixation could have on the amount of available antigenic sites (Fig. 4.8 A). Furthermore, lower labelling densities were found on osmium tetroxide treated samples compared to the sections fixed with glutaraldehyde (data not shown). Osmium tetroxide or glutaraldehyde treated sections had the same distribution of labelling (Fig. 4.7). Therefore the possible non-specific labelling due to charging of the sections, did not occur in this study (Stirling, 1995).

Osmium treated sections preserved the membranes clearly. The clear visibility of membranes on the osmium treated sections made it possible to deduce a transportation route for the β-1,3-glucanases. The labelling present in the cytoplasm seemed to be localised to the Golgi apparatus and endoplasmic reticulum (ER), which is the transportation route for protein targeted extracellularly (Fig 4.7 A). The β-1,3-glucanase pro-peptides enter the export pathway (Golgi and ER) and along the way, protein processing is completed. β-1,3-glucanases are synthesized as larger precursors with a N-terminal signal peptide (Shinshi *et al*., 1988; Vögeli *et al*., 1988). The signal peptides are responsible for the allocation thereof to cellular components like the intercellular space or vacuole (Chrispeels, 1991; Raikhel *et al*., 1993). Pihakaski-Maunsbach *et al*., (1996) and Kang and Buchenauer (2002) validated the low amounts of labelling found in the cytoplasm as being on-route to the apoplast.

β-1,3-glucanase accumulation in the different cellular compartments would lead to an increase in the β-1,3-glucanase activity. The sectioned material had an increase in β-1,3-

glucanase activity (Fig. 4.9). This increase could therefore be seen as an increase in the amount of labelling due to increases in the amount of antigenic sites present.

The accumulation of β-1,3-glucanases and chitinases in tomato plants in response to fungal infection was already documented in 1976 by Pegg. After the initial discovery, many other studies have implicated these two enzymes in the plant's defences against pathogens. It was shown that these enzymes had the ability to attack and degrade isolated fungal cell walls (Young and Pegg, 1982; Schlumbaum *et al.*, 1986; Mauch *et al.*, 1988a). However, the only conclusive evidence connecting β-1,3-glucanases and chitinases with the defence of plants against pathogens was the precise *in situ* localisation of the enzymes in fungus-infected plant tissues (Benhamou *et al.*, 1989).

In resistant and susceptible tomato plants infected with vascular wilt fungi a similar accumulation of β-1,3-glucanase labelling was found, with very high labelling present in the resistant plants (Benhamou *et al.*, 1989). In susceptible tomato plants, the enzyme accumulation was found to occur because of successful tissue colonization, whereas it appeared to be an early event associated with the limited spread of the fungus in resistant tissues. Therefore, in susceptible plants only cell walls of invaded cells were labelled while uninvaded cell walls of resistant plants displayed intense labelling. The proximity of the expressed β-1,3-glucanases to the invading pathogen and its early induction in the resistant plants demonstrated its possible function in the defence against some pathogens. The localisation of the accumulated β-1,3-glucanases in resistant and susceptible wheat plants infested with RWA could also demonstrate such a possible function.

The RWA is a phloem feeder and is able to probe intercellularly until the phloem is located where feeding commences (Pollard, 1973). It is therefore not surprising to find a differential induction in labelling in the vascular bundle cell walls, mesophyll cell walls and mesophyll chloroplasts (Fig. 4.16). All of these tissues or cellular components which showed increased labelling are therefore those parts of the wheat leaves which are most affected by the probing aphid.

The vascular bundle is the main objective of the probing aphid. To locate the vascular bundle the aphid probes intercellularly through the mesophyll cell walls and it was shown that the chloroplasts adjacent to the stylet path show early damage in barley plants infested with RWA (Belefant-Miller *et al.*, 1994).

The vascular bundle and mesophyll cell wall labelling are in agreement with β-1,3-glucanase localisation studies of plants infected with different pathogens. Wheat plants infected with *Fusarium culmorum* had high labelling densities mainly extracellularly with very little labelling on the cytoplasm or plant organelles (Kang and Buchenauer, 2002). Cell walls of infected resistant plants had significantly higher labelling densities compared to that of uninfected plants. A high density of gold particles was found on secondary thickenings of the xylem vessels of resistant plants with lower quantities in susceptible plants. Hu and Rijkenberg (1998) employed a β-1,3-glucanase antiserum to investigate the ultrastructural localisation of this enzyme during the compatible and incompatible interaction of *Puccinia recondita* in wheat leaves. They found low labelling in the compatible interaction on the secondary thickening of xylem vessels, in the cell walls of phloem elements and on the plasmalemma. In the incompatible interaction, the labelling was much higher and β-1,3-glucanases accumulated in the mesophyll cell plasmalemma close to the fungus. Increased labelling was also found in the extracellular matrix and on the secondary thickenings of xylem vessels.

In contrast to these results, Mauch *et al*., (1992) found that β-1,3-glucanases induced by ethylene treatment accumulate predominantly in the vacuoles with minor labelling in the middle-lamellae of the cell wall. Benhamou *et al*., (1989) considered the possibility that antibodies used for β-1,3-glucanase detection could show different affinities. Using an antiserum raised against a purified acidic β-1,3-glucanase might not have an affinity for the basic β-1,3-glucanases usually localized in the vacuole (Dore *et al*., 1991). This could explain the differences in labelling obtained using an antibody raised against a basic β-1,3-glucanase in the ethylene treated bean leaves. All the other localisation studies where different labelling was obtained used acidic anti-glucanase antibodies.

Chloroplast labelling in our study was unexpected because the presence of β-1,3-glucanases in chloroplasts has not yet been reported. Kang and Buchenauer (2002) found very low labelling on nuclei, Golgi bodies, mitochondria and chloroplasts on *Fusarium culmorum*-infected wheat spikes, but dismissed it as random labelling. In this investigation, the labelling in the chloroplasts was however significantly higher than what would be expected of random labelling alone while the labelling density increased in the resistant plants after infestation (Fig. 4.16 C). The pre-immune serum did not cause any significant labelling on the sections (Fig. 4.10 A to F), a further indication that the labelling is not due to random labelling.

Hu and Rijkenberg (1998) who used the same antibody to localise β-1,3-glucanase in wheat infected with *Puccinia reconditia* f.sp. *tritici* did not find any labelling on the chloroplasts. It would be expected that if the chloroplast labelling during our study were due to random labelling, they would also have had labelling in the chloroplasts. Furthermore, non-specific labelling would be expected to be present on all the sections from infested and uninfested susceptible and resistant plants. There is however, a selective increase in labelling present only in the infested resistant plants. This increase was not only in the chloroplasts, but also in the vascular bundle and mesophyll cell walls. These results correlate with the localisation work done on pathogen infection (Benhamou *et al*., 1989; Kang and Buchenauer, 2002). Random labelling would not be expected to show any such correlations.

The fact that nobody previously reported the localisation of β-1,3-glucanases in the chloroplasts prompted us to verify the results. The chloroplasts of uninfested and infested susceptible and resistant wheat plants were isolated and fractionated into three fractions (Fig. 3.1). These fractions were tested for β-1,3-glucanase activity and cross-reaction with the β-1,3-glucanase antibodies. The results obtained (Fig 4.17; Fig. 4.18) verified the presence of a β-1,3-glucanase in the chloroplasts. The β-1,3-glucanase activity, as well as the 35 kDa peptide detected with the β-1,3-glucanase antibody resided in the chloroplast membrane fraction which included the grana membranes. This was confirmed by the labelling that was localised in the grana of the chloroplast (Fig. 4.14 D and F; 4.15 C to F).

Proteins targeted to the chloroplasts usually have a characteristic signal peptide channelling them to the chloroplast (Kuchler and Soll, 2001). However, Grimm *et al.*, (1989) found a 22 kDa heat shock protein encoded by the nuclear genome that was transported to the chloroplasts without having a cleavable targeting signal. This might indicate that β-1,3-glucanase could also be targeted to the chloroplasts without having a visible targeting signal.

Van den Bulcke *et al*., (1989) found that *Pseudomonas syringae* infected and salicylic acid treated tobacco plants induced different extracellular β-1,3-glucanase isoforms due to the different stimuli. Normal growing tobacco plants accumulated β-1,3-glucanases in the vacuole and these vacuolar β-1,3-glucanases were not secreted extracellularly upon infection. It would therefore seem that these β-1,3-glucanase isoforms are governed by a set of distinct regulatory mechanisms. They further proposed the possibility that the specific elicitor could play a role in the accumulation of the β-1,3-glucanases at strategic sites where the PR protein

is needed in the defence against the particular pathogen. Fouche *et al.*, (1984) gave evidence that the primary site of feeding damage in susceptible wheat appears to be the chloroplasts. Ultrastructural studies showed that the chloroplasts of infested resistant plants were unaffected, while chloroplasts of susceptible plants were damaged at a very early stage of infestation. The question arises as to whether β-1,3-glucanase could play any role in the prevention of chloroplast damage, thereby contributing to the survival of the resistant cultivars.

Hincha *et al*., (1997) have shown that β-1,3-glucanases prevented damage to thylakoid membranes *in vitro* during thawing. Furthermore, spinach and cabbage leaves accumulated a class I β-1,3-glucanase under cold stress. β-1,3-glucanase accumulates in the cold hardening of wheat at 2ºC (Gaudet *et al*., 2000). Whether β-1,3-glucanases actually do play a role in the protection of chloroplasts *in vivo* remains to be proven. Localisation of glucanase-like proteins (GLPs) during cold stress had the same extracellular labelling pattern as β-1,3-glucanase after RWA infestation, however no chloroplast labelling was found. These GLPs are thought to help in the prevention of damage to cells during cold stress (Pihakaski-Maunsbach *et al*., 1996). β-1,3-glucanase could therefore have a similar protective role in the chloroplasts.

β-1,3-glucanases would seem to be favourable to the aphid since it has the ability to hydrolyse callose, which permits continued flow of nutrients in the phloem (Forslund *et al*., 2000). On the other hand, the hydrolysed callose could also be responsible for callose turnover, which in turn is responsible for the callosic barrier surrounding the point of insect attack (Bowles, 1990; Bronner *et al*., 1991). The cell wall associated β-1,3-glucanases could also be responsible for the release of oligosaccharides from the plant cell wall that could act as could elicitors, which trigger defence reactions in plants (Dixon and Lamb, 1990; Bergey *et al.*, 1999). By investigating the localisation of the expressed β-1,3-glucanase after RWA infestation, the expectation was to assign a role to the β-1,3-glucanase by observing its localisation. Inbar *et al*., (1998) concluded that the expressed β-1,3-glucanases have no apparent effect on insects. However, β-1,3-glucan binding proteins have been found in insects. These proteins, which bind to β-1,3-glucans, activates the prophenoloxidase cascade to protect the insect against yeast and Gram negative bacteria (Beschin *et al*., 1998). Lipopolysaccharide, peptidoglycan and β-1,3-glucan are all biologically active and elicit various innate immune reactions such as septic shock, cytokine synthesis and acute-phase

protein synthesis in both insects and mammals (Ochiai and Ashida, 2000). The extracellular β-1,3-glucanases would release large amounts of β-1,3-glucan which would be ingested by the aphid and possibly elicit the immune response.

The expression of PR proteins in the resistance response indicates that if these PR proteins can be expressed before pathogen infection occurs, these plants would be resistant. Benzothiadiazole (BTH), which is a synthetic inducer of PR protein synthesis, has the ability to induce resistance in susceptible plants. Godard *et al*., (1999) induced resistance to *Peronospora parasitica* in susceptible cauliflower plants by spraying it with BTH. Another important inducer is salicylic acid. It plays a central role in a plant's ability to activate systemic acquired resistance, which is part of the HR (Delaney *et al*., 1994). When pea plants were sprayed with salicylic acid, it induced systemic resistance to *Erysiphe pisi* (Frey and Carver, 1998). An alternative to spraying plants with chemicals to induce resistance is to transform susceptible plants with PR protein genes. Most of these transformed plants have shown increased resistance against pathogens (Jach *et al*., 1995). Plants transformed with both chitinase and β-1,3-glucanase genes had increased resistance while on their own these PR proteins were not as effective (Jach *et al*., 1995). The transformation of wheat plants with PR proteins, could be used to induce resistance in susceptible wheat cultivars. This would eliminate the time consuming breeding of resistance by conventional breeding methods.

To conclude: The different resistance genes affected the expression of β-1,3-glucanase. The Dn1 and Dn2 resistance gene containing plants showed similar induction of β-1,3-glucanase genes. Plants with the Dn5 resistance gene had much lower β-1,3-glucanase protein expression. This might indicate an important different β-1,3-glucanase expression mechanism with the Dn5 resistance gene that function independently of the other two. Northern blots indicated that the expression of the β-1,3-glucanase proteins is regulated at the level of transcription.

The localisation studies revealed that these β-1,3-glucanase proteins accumulated where tissues were affected most by feeding aphids. Aphids mainly probe intercellular and penetrate the phloem to feed. The vascular bundle cell walls had very high labelling density. The chloroplast damage inflicted during aphid feeding is eliminated in resistant plants, which have high levels of labelling. The role of β-1,3-glucanase during the resistance response of

Diuraphis noxia is not clear. However, it could play a protective role in the chloroplasts and activate the aphid's own defences.

SUMMARY

The aim of the study was to learn more about the expression of β-1,3-glucanase during the resistance response of wheat to the RWA (*Diuraphis noxia*)(Mordvilko).

The different resistance genes (Dn1, Dn2 and Dn5) used in the study would shed more light on the possibility that β-1,3-glucanase expression is a universal biochemical resistance mechanism with regard to RWA. Tugela DN (Dn1) and Dn2 plants resulted in a similar expression pattern of β-1,3-glucanases after RWA infestation. The Dn1 and Dn2 cultivars had very high differential β-1,3-glucanase activity induction, which occurred much sooner compared to the infested susceptible plants. Wheat plants with the Dn5 resistance gene induced β-1,3-glucanase activity to a much lower level compared to the other two resistant lines.

Northern blot analyses confirmed that the increase in β-1,3-glucanase activity and peptide levels in the Western blot analyses was due to increased transcription of β-1,3-glucanase genes.

The induced β-1,3-glucanases were located intercellularly in the vascular bundle and mesophyll cell walls of wheat leaves. Intracellular the β-1,3-glucanases were primarily located in the chloroplast grana with low labelling found in the cytoplasm.

Chloroplasts were isolated and fractionated to scrutinize results obtained with the localisation study. β-1,3-glucanase activity and Western blot analysis of chloroplast fractions verified these results with a 35 kDa β-1,3-glucanase residing in the chloroplast membrane bound fraction. These β-1,3-glucanases could play a role in the protection of the chloroplast of infested resistant plants.

OPSOMMING

Die doel van die studie was om meer lig te werp op die uitdrukking van β-1,3-glukanase gedurende die weerstandsrespons van koring teen die Russiese koringluis (RKL) (*Diuraphis noxia*)(Mordvilko).

Die verskillende weerstandsgene wat in hierdie studie gebruik is, sal meer duidelikheid gee of β-1,3-glukanase uitdrukking deel is van ‘n universele biochemiese weerstandsmeganisme teen die RKL. β-1,3-glukanase aktiwiteit is dieselfde in Tugela DN en Dn2 plante, deur RKL-infestering geïnduseer met die induksie wat vroeër plaasgevind het as in die geïnfesteerde vatbare plante. Koringplante met die Dn5 weerstandsgeen se β-1,3-glukanase-aktiwiteit was tot baie laer vlakke geïnduseer as die ander twee weerstandbiedende kultivars.

Northern kladanalises het getoon dat die toename in β-1,3-glukanase-aktiwiteit en polipeptied vlakke soos gesien in die Western kladanalises, die gevolg van ‘n toename in transkripsie van β-1,3-glukanasegene was.

Die geïnduseerde β-1,3-glukanaseproteïne was veral in die vaatbondel- en mesofielselwande gelokaliseer. Intrasellulêr, was die β-1,3-glukanase in die chloroplaste gelokaliseer en lae vlakke het in die sitoplasma voorgekom.

Om die chloroplast lokalisering van β-1,3-glukanase te bevestig, is chloroplaste geïsoleer en in drie fraksies gefraksioneer. Die β-1,3-glukanase aktiwiteit en Western kladanalises van die chloroplastfraksies het op die teenwoordigheid van β-1,3-glukanase in die chloroplaste gedui. In die chloroplasmembraanfraksie was ‘n 35 kDa β-1,3-glukanase teenwoordig wat die teenwoordigheid van β-1,3-glukanase in die chloroplaste van koringplante bevestig. Hulle mag moontlik ‘n rol speel in die beskerming van weerstandbiedende plante se chloroplaste.

CHAPTER 6

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