Pathogenesis-Related Proteins in Barley

Localization and accumulation patterns in response to infection by *Bipolaris sorokiniana*

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Doctoral thesis
Swedish University of Agricultural Sciences
Alnarp 2007

Acta Universitatis Agriculturae Sueciae
2007: 86

ISSN 1652-6880
ISBN 978-91-576-7385-5

Cover photo: Kerstin Brismar
Tryck: SLU Service/Repro, Alnarp 2007

Abstract

Santén, K. 2007. *Pathogenesis-related proteins in barley. Localization and accumulation patterns in response to infection by* Bipolaris sorokiniana. Doctoral dissertation.
ISSN 1652-6880, ISBN 978-91-576-7385-5

Plants in nature are generally resistant to most of the pathogens they encounter. However, many fungal pathogens can cause severe diseases and significant yield losses in crops. Plants defend themselves against pathogens through a combination of constitutive and inducible defenses. The induced plant defense is particularly characterized by an increased accumulation of pathogenesis-related proteins (PRs). Since the discovery of PRs in 1970, several PR families have been identified. The specific function of many PRs is still unknown, although several are postulated to play a role in preventing pathogen invasion.

The aim of my research was to study spatial and temporal localization of PRs and accumulation of their corresponding mRNAs to better understand the role and possible function of PRs in plant defense. As a model system I studied the interaction between barley and the hemibiotrophic fungus *Bipolaris sorokiniana*, which causes severe yield losses worldwide.

The studies are mainly focused on three PRs (PR-1, PR-3 and PR-5). These PRs accumulated in both leaves and roots of barley seedlings as a response to infection of the respective tissues. However, the accumulation of PRs in roots was dependent on whether young or old root segments were infected. A stronger response was found when young root segments were inoculated in comparison to old roots. All three PRs appear to be part of a preformed defense since both PR-1 and PR-5 were constitutively present in both xylem and phloem tissues and the root epidermis, and PR-3 showed a high constitutive presence mainly in the epidermis of leaves and to some extent in the phloem. In response to infection, all three proteins were highly induced in the ground tissues and to some extent in the vascular tissues. PR-1, PR-3 and PR-5 mRNAs accumulated in a biphasic pattern in leaves from both leaf- and root-infected seedlings. This biphasic accumulation pattern of transcripts was not detectable in roots. These results suggest that different induction strategies may be active in barley seedlings depending on the primary site of infection by *B. sorokiniana.*

PR-1, PR-3 and PR-5 were all recovered over the cell walls of inter- and intracellular hyphae of *B. sorokiniana* indicating that they may influence fungal growth.

Keywords: *Pathogenesis-related proteins, PR-1, PR-3, PR-5, plant defense, barley* (Hordeum vulgare), Bipolaris sorokiniana, *immunolocalization, microscopy.*

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To Stefan, Oskar and Alice

&

Iris and Sven

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List of Appendices

Papers

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I Liljeroth, E., Santén, K., and Bryngelsson, T. 2001. PR protein accumulation in seminal roots of barley and wheat in response to fungal infection - the importance of cortex senescence. *Journal of Phytopathology 149*, 447-456.

II Santén, K., Marttila, S., Liljeroth, E., and Bryngelsson, T. 2005. Immunocytochemical localization of the pathogenesis-related PR-1 protein in barley leaves after infection by *Bipolaris sorokiniana*. *Physiological and Molecular Plant Pathology 66*, 45-54.

III Santén, K., Liljeroth, E., Bryngelsson, T., and Marttila, S. Comparison of spatial and temporal accumulation of PR-1 and PR-5 in relation to infection site in barley infected by *Bipolaris sorokiniana*. (Manuscript).

IV Santén, K., Bengtsson, T., Liljeroth, E., Bryngelsson, T., and Marttila, S. Differential localization and gene expression of PR-3 in barley leaf and root in response to infection by *Bipolaris sorokiniana*. (Manuscript) .

1. Introduction

Death by disease is an exception rather than a rule in plants. So far about 100 000 species of fungi are known, of which approximately 50 species cause diseases in humans and more than 10 000 species can invade plants (Agrios, 1997). A fungal disease in plants is usually caused by only one species; however, a fungal species can attack one or several plant species. Still, completely successful colonization occurs only in limited cases.

Plants have no immune system, though; coevolution of plants and pathogens has created a multifaceted relationship, resulting from the exchange of molecular information between the species (Benhamou, 1996). Based on this, plants have developed a complex surveillance system with an array of defense mechanisms. Pathogens, on the other hand, possess strategies to overcome the defense system and colonize plants. The difference between success and failure of plant defense is most likely dependent on the time it takes for the plant to recognize a potential pathogen and subsequently activate the defense system (Garcia-Brugger *et al*., 2006). Once the defense is alarmed a wide range of proteins is induced in the plant; among these the pathogenesis-related proteins (PRs) (van Loon *et al*., 2006). The specific function of many PRs is still unknown although several of them are postulated to play a role in preventing pathogen invasion (Table 1).

I have studied the accumulation and localization of some of these PRs that may shed some light on their possible functions. The interaction between barley (*Hordeum vulgare*, L.) and *Bipolaris sorokiniana* was chosen as model system (Fig. 1). Barley is the fourth largest cereal crop grown in the world today (FAOSTAT, 2005). Depending on quality, barley grains are most commonly used for feed and beer or whisky malt. In Sweden around 1.6 million ton is harvested per year, which is almost 1.2% of the world production (FAOSTAT, 2005). The pathogen *B. sorokiniana* is a worldwide spread fungus that causes severe economical losses in cereal crops each year (Kumar *et al*., 2002). *B. sorokiniana* is also a valuable model fungus as it can infect both below and above ground parts of cereals, which allows studies of defense mechanisms in the whole plant.

Figure 1. Left. *Field of barley* (© *K. Brismar*). **Center**. *Distribution map of* B. sorokiniana *1986* (© *CAB*). **Right**. *Barley leaf infected by* B. sorokiniana (© *E. Liljeroth*).

2. Background

2.1 Plant defense

Leaves and roots are target organs for several pathogens. To survive plants have developed a number of coordinated defense responses, not just relying on an altered expression of a few unique defense-related genes but on an extensive change in gene activity (Yang *et al*., 1997).

The defense is based on preformed barriers and induced responses (Bryngelsson & Collinge, 1992). Preformed barriers are for example the cuticle, host cell walls and antimicrobial compounds. The inducible response, initiated after a pathogen has tried to adhere onto a host surface, involves the recognition of specific signal molecules. These originate either from the pathogen or from degradation products of the host cell walls, and are termed exogenous or endogenous elicitors, respectively (Fig. 2) (Collinge *et al*., 1993; Fujita *et al*., 2004). The elicitors appear to trigger a network of signaling pathways to coordinate the succeeding defense responses (Yang *et al*., 1997). Numerous nuclear genes are activated or repressed (Hammond-Kosack & Jones, 1996; Yang *et al*., 1997). Early and rapid recognition of a pathogen is thus a crucial step for the plant since it ultimately leads to a fast activation of defense response genes. Resistance in plant species is often divided into host or non-host specific. Host-specific resistance is expressed in interactions between specific host and pathogen genotypes, which gives a pathogen race-specific resistance. Non-host resistance, shown by a whole plant species against a specific parasite or pathogen, is the most common form of resistance in plants towards the majority of potential pathogens (Heath, 2000). The biochemical changes that occur during infection are very similar in host- and non-host resistant plants (Somssich and Hahlbrock, 1998).

The earliest defense responses are the opening of specific ion channels across the plasma membranes, the rapid production of active oxygen species (AOS), such as O_2^- and H_2O_2, known as the oxidative burst, and phosphorylation and dephosphorylation of specific proteins (Doke *et al*., 1996; Conrath *et al*., 1997). These initial reactions are a prerequisite for initiation of the signaling network that will trigger the overall defense response (Hammond-Kosack & Jones, 1996). AOS can be toxic to pathogens per se but can also lead to the so-called hypersensitivity response (HR), a localized and rapid death of one or a few cells at the infection site, to restrict further invasion. HR may present resistance to biotrophic pathogens that obtain their energy from living cells, but in the case of necrotrophs, that obtain their energy from dead cells, cell death may be beneficial to the pathogen (Kumar *et al*., 2001). Following activation of HR, uninfected distal parts of the plant may develop resistance to further infection, a phenomenon known as systemic acquired resistance (SAR) (Ryals *et al*., 1996). In several plant species, SAR is triggered by a systemic increase of salicylic acid (SA). Plants can activate separate defense pathways depending on the type of pathogen encountered (Garcia-Brugger *et al*., 2006). Jasmonic acid (JA)- and ethylene-dependent responses seem to be initiated by necrotrophs, whereas the SA-dependent response is activated by biotrophic pathogens. However, cross talk and synergistic effects between the defense

pathways mediated by SA, JA or ethylene during different pathogenic infections has been proposed (Thomma *et al*., 1998; Schenk *et al*., 2000).

All three pathways are associated with enhanced transcription of numerous defense genes. One major class of these genes encodes PRs that are massively induced both locally around infection sites and systemically (van Loon *et al*., 2006). The oxidative burst aids in cross-linking reactions and activation of enzymes involved in strengthening the plant cell walls and the accumulation of secondary metabolites, such as phytoalexins. Thickened cell walls or specific papilla formation at sites of penetration are dependent on the accumulation of compounds such as lignin, suberin, thionins, hydroxyproline-rich glycoproteins (HRGP) and polysaccharides such as cellulose, callose and pectins (Collinge *et al*., 1993; Brisson *et al*., 1994; Baker & Orlandi, 1995). It seems that various combinations of available defense mechanisms can create an efficient defense against most phytopathogens (Somssich & Hahlbrock, 1998).

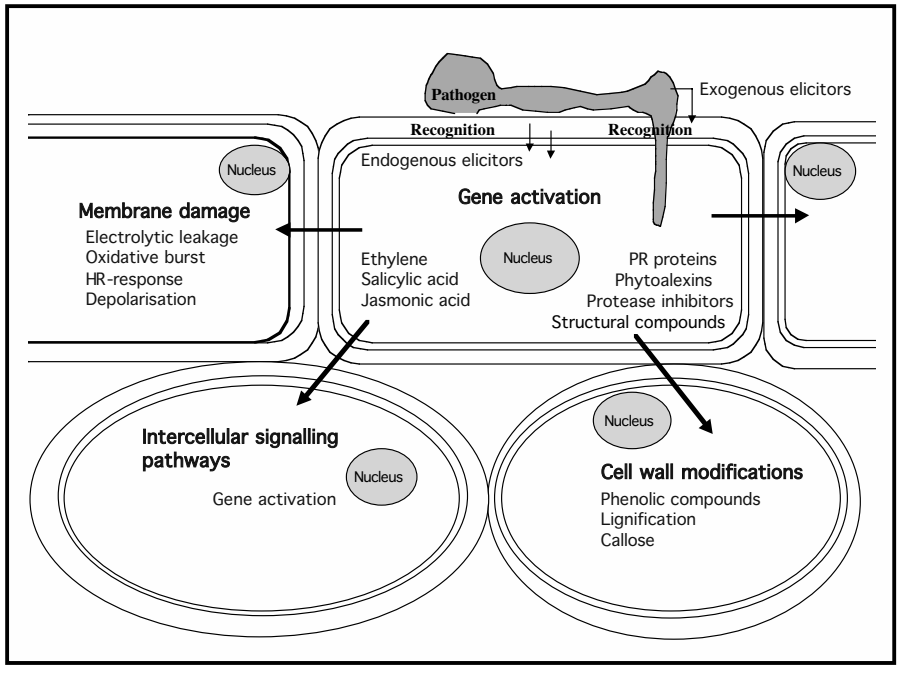

Figure 2. *Schematic representation of plant defense responses in plant-pathogen interactions* (*modified from Benhamou* et al., *1996*).

2.2 Pathogenesis-related proteins

PRs are usually defined as host-specific proteins that are induced in several, if not all, plant species during pathological or related situations (van Loon *et al*., 2006). PRs accumulate after pathogen attack by virus, viroids, bacteria, fungi, nematodes, insects and herbivores as well as after wounding and certain abiotic stress conditions (van Loon *et al*., 2006). High amounts of PRs accumulate when plants respond with HR. PRs, absent or present at low concentrations in healthy plants, may within a few days after infection account for up to 10% of the soluble proteins in leaves (van Loon *et al*., 1987).

PRs are low molecular weight proteins (10-40 kDa), which can survive in harsh environments due to their biochemical properties. They are able to remain soluble and very stable at low pH where most other plant proteins are denaturated; they are relatively resistant against proteolytic cleavage; are predominantly localized in the vacuole, cell wall and the intercellular space and they usually have extreme isoelectric points (pI) (Stintzi *et al*., 1993).

Since the discovery of PRs in 1970, 17 PR families have been identified (Table 1) based on amino acid sequences, serological relationship and/or enzymatic or biological activity (van Loon *et al*., 1994, 2006). Within each PR family, there are several classes comprising of different isoforms with either high (basic) or low (acidic) pI values. Most of the families were originally identified from tobacco but some also from other plant species, including monocotyledons such as barley, wheat, rice and maize. The fact that PRs from different plant species have the same family designation does not necessarily mean that they are identical proteins. The families are numbered in the order in which they were discovered and new PRs identified in different species are assigned to the existing recognized families and, if no similarity exists, a new family is created.

The specific functions of PRs are not fully understood (Table 1). Although the term pathogenesis-related refers to the phenomenon that PRs are expressed in association with resistance responses it does not state that they have functional roles in defense (van Loon *et al*., 2006). PRs can be induced systemically in non-infected distant leaves as a result of a primary infection, which indicates a role in contributing to an enhanced level of protection (Ward *et al*., 1991).

Certain PRs are often referred to as defense proteins functioning in limiting the multiplication and spread of pathogens. There is evidence that various PRs have potential antimicrobial activity and are involved in defense mechanisms against some fungal pathogens (van Loon, 1997). Different isoforms of PRs, mainly basic, have been shown to exhibit antifungal activity and target specificity *in vitro*, especially when different PRs are combined to create synergistic effects (Niderman *et al*., 1995; Jacobsen *et al*., 1990). This indicates that a coordinated induction of different PRs may create a resistance considerably broader than any protein by itself. Some PRs might play a role as internal signal generating molecules involved in the localization and recognition of a potential pathogen. However, genetic engineering of plants to improve resistance against fungi and bacteria by transformation has so far reduced only a limited number of diseases, depending on the nature of the PR protein or proteins, plant species and pathogen involved (van Loon *et al*., 2006).

Barley with transiently silenced PR-1 expression showed that PR-1b is one of the factors that limits penetration of the leaves by the powdery mildew fungus (Schultheiss *et al*., 2003). In most plant species PRs are expressed in both incompatible and compatible reactions. Since PRs within different families are closely related and their mRNA and protein may readily cross-hybridize it is difficult to clarify a specific role in resistance for a single isoform (van Loon *et al*., 2006).

Table 1. *Recognized families of pathogenesis-related proteins (modified from van Loon* et al., *2006)*

Family	Type member	Properties
PR-1	Tobacco PR-1a	unknown
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase class I, II, IV-VII
PR-4	Tobacco R	Chitinase class I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato inhibitor I	Proteinase-inhibitor
PR-7	Tomato P_{69}	Endoproteinase
PR-8	Cucumber chitinase	Chitinase class III
PR-9	Tobacco lignin-forming peroxidase	Peroxidase
PR-10	Parsley “PR-1”	Ribonuclease-like
PR-11	Tobacco ”class V”chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate-oxidase-like
PR-17	Tobacco PRp27	Unknown

Note: Further details can be found at http://www.bio.uu.nl/~fytopath/PR-families.htm

PRs appear to be multifunctional proteins, as many of them are detected also during plant development and senescence (Hanfrey *et al*., 1996; Liljeroth *et al*., 2005; van Loon *et al*., 2006). They can accumulate during specific developmental stages and be induced in response to infection in the same organ (Shinshi *et al*., 1987). Some PRs may be constitutively expressed in some organs, such as flowers, and inducible in other organs, such as leaves (Memelink *et al*., 1990). The induction of PRs in the absence of a pathogen might indicate a more physically protective role of the cellular structures, in order to stabilize sensitive membranes or macromolecules (van Loon & van Strien, 1999). Whatever the specific functions of PRs, it would seem strange if a single PR protein was the determining factor in a plants defense response or development. That would inevitably make plants too vulnerable. Since genes for many different PRs are already present in plants, efforts to manipulate the signals that trigger the expression of PRs may be a more effective approach to enhance plant resistance.

So far, 12 PR families have been isolated from barley (Muthukrishnan *et al*., 2001; Christensen *et al*., 2002; van Loon *et al*., 2006; T. Bryngelsson, personal communication) (Table 2). In this thesis I have focused on PR-1, PR-3 and PR-5 in barley. All three PRs have been extracted from intercellular washing fluid from barley leaves infected by *Blumeria graminis* f. sp. *hordei* (Bryngelsson *et al*., 1994).

Table 2. *Pathogenesis-related proteins in barley (modified from Muthukrishnan* et al*., 2001)*

Family	Class	Induced by
PR-1	Basic	Pathogen
PR-2	Glucanase	Pathogen & developmental
PR-3	Chitinases I & II	Pathogen & developmental
PR-4	Chitin binding, hevein	Pathogen
PR-5	Thaumatin-like	Pathogen & developmental
PR-6	Thaumatin-like	Pathogen
PR-8	Chitinase III	Pathogen
PR-13	Thionin	Pathogen & developmental
PR-14	Lipid-transfer protein	Pathogen
PR-15	Oxalate oxidase	Pathogen
PR-16	Oxalate-oxidase-like	Pathogen
PR-17	Aminopeptidase-like	Pathogen

Note: Authors and references are available in Mutukrishnan et al*., 2001, except for PR-8 (T. Bryngelsson, personal communication) and PR-17 (Christensen* et al., *2002).*

2.3 PR-1

The PR-1 family contains the first discovered PRs and is also the most predominant. Despite extensive studies, no biochemical function is known for any of the PR-1 proteins (van Loon *et al*., 2006). Tobacco plants transformed to constitutively express PR-1 showed enhanced resistance specifically against two oomycete fungi, *Peronospora tabacina* and *Phytophthora parasitica* var *nicotinae*. However, the transformants were as susceptible as the non-transformants when challenged with other types of fungi, bacteria or viruses (Linthorst *et al*., 1989; Alexander *et al*., 1993). When aliquots of tobacco PR-1 were applied to leaf discs of tobacco, it significantly reduced the development of *Phytophthora infestans* and an additional *in vitro* study showed that especially a basic tobacco PR-1 exhibited negative effects on this oomycete (Niderman *et al*., 1995).

Reports on the localization of different PR-1 proteins in infected tissues show multiple localizations. PR-1 has been localized to vacuoles in tomato infected by citrus exocortis viroids and in tobacco after being induced by darkness (Vera *et al*., 1989; Sessa *et al*., 1995). In potato leaves infected by *P. infestans,* PR-1 was localized in epidermis including stomata guard cells and glandular trichomes, the intercellular space, crystal idioblasts and in both phloem and xylem tissues of vascular bundles (Hoegen *et al*., 2002). PR-1 proteins have also been found in cell walls of *Phytophthora capsici* and *Chalara elegans* (Tahiri-Alaoui *et al*., 1993; Hong & Hwang, 2002). A PR-1 protein in maize, PRm, has been localized specifically to the plasmodesmata of the phloem in both maize and transgenic tobacco plants (Murillo *et al*., 1997; Bortolotti *et al*., 2005). PR-1 gene transcripts

have been localized to special phloem cells in the vascular bundle of pepper stems infected with *P. capsici* (Lee *et al*., 2000a). Additionally, PR-1 has been detected in the xylem sap from non-infected *Brassica napus* and in the guttation fluid from non-infected barley seedlings, indicating long-distance transport of PR-1 in the transpiration stream (Grunwald *et al*., 2003; Kehr *et al*., 2005).

2.4 PR-2

The PR-2 proteins are β-1,3-glucanases, endoglucanases that can catalyze hydrolytic cleavage of β-1,3-D-glucosidic linkages in β-1,3-glucans (Leubner-Metzger & Meins, 1999). PR-2 is believed to act primarily on glucans present in the cell wall of most fungal pathogens to release oligosaccharides (Mauch & Staehelin, 1989). The plant may then perceive these fragments as elicitors that serve to trigger further defense responses. PR-2 is also active in plant reproductive processes and ripening of fruits (Leubner-Metzger & Meins, 1999).

The PR-2 family is divided into three structurally distinct classes of β-1,3-glucanases, with acidic and basic counterparts that significantly differ in their specific enzymatic and antifungal activity (Kauffmann *et al*., 1987; Sela-Buurlage *et al*., 1993). Several *in vitro* experiments have demonstrated antifungal effects mainly by basic class I β-1,3-glucanases against a wide range of fungi, either alone or in combination with PR-3 (Mauch *et al*., 1988; Ludwig & Boller, 1990; Sela-Buurlage *et al*., 1993). The synergistic effect between PR-2 and PR-3 has also been shown in transgenic plants (Zhu *et al*., 1994). Morphological studies on the influence of PR-2 on hyphal tips of *Trichoderma longibrachiatum* showed that PR-2 and PR-3 together are particularly effective at the hyphal tip causing balloon-like swelling and lysis of the tip (Mauch *et al*., 1988; Arlorio *et al*., 1992). Both PR-2 and PR-3 are likely to play a dual role in plant defense both directly by hydrolyzing structural components from fungal cell walls and indirectly by releasing elicitors that may amplify the defense response in the plant (Stintzi *et al*., 1993).

PR-2 induced by ethylene has been restricted to the vacuoles of lower epidermal cells and parenchyma cells adjacent to vascular bundles and over the middle lamella in the intercellular space in bean leaves (Mauch & Staehelin, 1989; Mauch *et al*., 1992). In an incompatible reaction in wheat leaves against *Puccinia recondita*, PR-2 was mainly recovered in the domain of the host cell wall closest to plasmalemma, cell wall appositions, intercellular space, guard cells and secondary thickening of xylem vessels as well as in the hyphal cytoplasm and cell wall (Hu & Rijkenberg, 1998). Tomato roots infected with *Fusarium oxysporum* showed PR-2 predominantly localized in the cell walls and vacuoles of the host, and in the cell wall and septa of the fungus. Thickened secondary cell walls of the xylem vessels were also heavily labeled (Benhamou *et al*., 1989). PR-2 has been studied in floral organs of barley where it is developmentally regulated. It was localized in the anther and pistil tissues, including the stigmatic hairs. Besides cell walls, PR-2 was also recovered in plastids in cells of the style and ovary cell wall (Liljeroth *et al*., 2005).

2.5 PR-3

Proteins of the PR-3 family are endochitinases, which hydrolyze β-1,4-linkages between N-acetylglucosamines of chitin, releasing oligosaccharides from the cell walls of many fungi (Boller, 1993). Chitin is not a natural component of plant cells but is present in most fungal cell walls and the insect cuticula (Stintzi *et al*., 1993).

The division of chitinases into different classes (I-VII) is mainly based on the presence or absence of a cysteine-rich domain and of a C-terminal extension providing a signal for vacuolar targeting. The cysteine-rich domain is believed to be the chitin-binding part targeting PR-3 to chitin-containing pathogens (Neuhaus, 1999). Class I chitinases contain a cysteine-rich domain and has a 10-15 fold higher chitinase activity than class II chitinases that lack this domain (Sela-Buurlage *et al*., 1993). The high chitinase activity of PR-3 class I is also reflected in the antifungal activity demonstrated in *in vitro* studies of *Fusarium solani* and *Rhizoctonia solani* (Broglie *et al*., 1991; Sela-Buurlage *et al*., 1993). In fact, PR-3 has been shown to inhibit growth of most fungi but not of the oomycetes *Phytophthora* and *Pythium*, which lack chitin in their cell wall (Mauch *et al*., 1988). Several studies have also shown the synergistic effect of PR-3 and PR-2 as mentioned above. It has been proposed that the thinning of the fungal cell wall by PR-2 exposes the chitin present in the inner parts of the wall, making it accessible to chitinases to hydrolyze the fungal cell wall as well as to release elicitors (Kombrink & Somssich, 1997).

PR-3 has been localized to the vacuole of ethylene-treated bean leaves and tomato leaves infected with *Cladosporium fulvum* (Mauch *et al*., 1992; Wubben *et al*., 1992). Wheat spikes infected with *Fusarium culmorum* showed PR-3 mainly on the cell walls, over cell wall appositions, intercellularly and on cell walls of the hyphae (Kang & Buchenauer, 2002). PR-3 has also been localized specifically to the cell wall of the style and stigmal branches of flower organs in barley early in the development. The labeling intensity of PR-3 did however decrease significantly during the later stages of flower development (Liljeroth *et al*., 2005). Chitinase labeling has been reported on host cell walls and in the intercellular space of pepper stems infected with *P. capsici*, including the presence of chitinase mRNA in phloem-related cells (Lee *et al*., 2000b). The presence of chitinase mRNA in phloem cells has also been described in potato leaves challenged with *P. infestans* and in pepper leaves infected with *Colletotrichum coccodes* (Büchter *et al*., 1997; Hong & Hwang, 2002). Xylem sap from tomato infected with *F. oxysporum* revealed that the presence of PR-3 was very low in the fluid compared to PR-1, PR-2 and PR-5 (Rep *et al*., 2002). In contrast, chitinase-antifreeze proteins (AFP) induced by cold acclimation of rye were found in cell walls of all leaf tissues particularly abundant in epidermal cell walls and xylem vessels. The corresponding mRNAs were found in the same cell types as the chitinase-AFPs and in vascular parenchymal cells surrounding xylem vessels (Pihakaski-Maunsbach *et al*., 2001).

2.6 PR-5

Proteins that belong to the PR-5 family are also known as thaumatin-like (TL) proteins as they show sequence similarities to the sweet-tasting plant protein thaumatin (Linthorst, 1991). Osmotins, proteins induced by salt stress, also belong to the PR-5 family (Velazhahan *et al*., 1999).

Like other PRs the PR-5 proteins constitute of acidic-neutral and basic isoforms. In some dicotyledonous plants the extracellular PR-5 proteins tend to be acidic while the vacuolar ones tend to be basic. The various isoforms of PR-5 are associated with diverse functions such as antifungal activity, protection against osmotic stress (Kononowicz *et al*., 1992) and freezing tolerance (Hon *et al*., 1995). Several PR-5 proteins display significant activity *in vitro* in inhibiting hyphal growth, spore germination or development of germ tubes, probably by a fungal plasma membrane permeabilizing mechanism (Velazhahan *et al*., 1999). A basic barley PR-5 possesses inhibitory activity *in vitro* against germ tube development of *Blumeria graminis* (Tandrup Poulsen, 2001). A basic PR-5, osmotin from tobacco, has been shown to inhibit growth of *P. infestans, Neurospora crassa, Trichoderma reesei* and *Candida albicans in vitro* (Woloshuk *et al*., 1991, Vigers *et al*., 1992). Interestingly, two basic barley PR-5 proteins inhibited growth of *Trichoderma viride* and *C. albicans* (Hejgaard *et al*., 1991). However, one of their homologous acidic tobacco PR-5 did not show any activity against these fungi. Instead it was most potent against *Cercospora beticola* (Vigers *et al*., 1992). Tobacco osmotin induced spore lysis, inhibited spore germination or reduced spore viability in different species of *Bipolaris, Fusarium* and *Phytophthora*. However, the hyphal growth of *Aspergillus, Rhizoctonia* and *Macrophomina* was not affected by osmotin (Abad *et al*., 1996).

The first leaf of transgenic barley plants, with a pathogen-inducible epidermis-specific promoter fused to a basic PR-5, showed enhanced resistance against *B. graminis*, *Rynchosporium secalis* and *Drechslera teres*, while no disease reduction was observed on infection with *Puccinia hordei* (Tandrup Poulsen, 2001). Overexpression of PR-5 in potato delayed development of disease symptoms of *P. infestans* (Liu *et al*., 1994), whereas transgenic potato plants expressing antisense PR-5 did not exhibit any higher susceptibility (Zhu *et al*., 1996). Tobacco plants constitutively overexpressing a rice PR-5 showed enhanced resistance to *Alternaria alternata* (Velazhahan & Muthukrishnan, 2003). Overexpression of a specific fungal cell wall protein in *Saccharomyces cerevisiae*, otherwise susceptible to tobacco osmotin, increased the resistance, whereas deletion of the genes in a tolerant strain, resulted in sensitivity towards tobacco osmotin (Yun *et al*., 1997). The resistance or susceptibility of different fungi towards the different PR-5 isoforms indicates specificity in recognition between potentially antifungal proteins and certain binding features of different fungal cell wall proteins (Vigers *et al*., 1992; Yun *et al*., 1997).

Few localization studies have been reported with PR-5. A basic PR-5 was found on the cell wall of *P. infestans* and in starch granules of chloroplasts and in papilla of tomato leaves expressing SAR (Jeun & Buchenauer, 2001). In barley, PR-5 mRNA was specifically expressed in the mesophyll early after infection with the necrotrophic fungus *Rhynchosporium secalis* (Steiner-Lange *et al*., 2003). It has been shown that PR-5 proteins are among the most abundant proteins in the xylem sap of healthy *Brassica napus* (Kehr *et al*., 2005).

2.7 The pathogen Bipolaris sorokiniana

Bipolaris sorokiniana is an economically important fungal pathogen that affects cereal crops worldwide. The fungus also has a wide range of wild grasses as potential hosts (Bakonyi *et al*., 1998). Diseases caused by this fungus result in significant yield losses in warmer climate regions. However, it is distributed in all the major cereal growing regions of the world. A field that does not contain *B. sorokiniana* is rarely found (Kumar *et al*., 2002; Mathre *et al*., 2003).

Figure 3. *A conidium of* B. sorokiniana *with bipolar germination on a barley leaf (© E. Liljeroth). Bar 50 μm.*

B. sorokiniana has the ability to cause several diseases on plant tissues both above and below ground (Agrios, 1997). The fungus causes infections on aerial plant parts under warm and humid conditions while during dry conditions infection occurs mainly below ground (Sivanesan & Holliday,1981). Besides causing leaf spot blotch, head blight and black point in seeds, *B. sorokiniana* is also the casual agent for seedling blight, crown and common root rot (Kumar *et al*., 2002; Duveiller & Altamirano, 2000). Although both leaf spot blotch and common root rot may occur simultaneously, one disease form usually prevails over the other, due to the climatic conditions (Duveiller & Altamirano, 2000). Seeds, infested soils and host debris on the soil surface can transmit inocula of *B. sorokiniana* (Agrios, 1997).

When favored by warm and moist climate, *B. sorokiniana* can cause spot blotch, one of the most serious foliar diseases in cereals, particularly in South Asia, China, North and Latin America (Kumar *et al*., 2002). At higher altitudes, such as the Canadian prairies, the great plains of the United states, parts of Australia and in the Russian federation, *B. sorokiniana* is the dominant pathogen causing common root rot and seedling blight (Piening, 1997; Tinline *et al*., 1988). Thus far, *B. sorokiniana* has been regarded less important in Northern Europe, although attacks on barley have been reported. The infection level of barley seeds in Southern Sweden is normally 2-5%, up to 14-41% in susceptible cultivars (Luttenberger, 1992).

Taxonomy

B. sorokiniana (Sacc. in Sorok) Shoem. (syn. *Helminthosporium sativum*) is the anamorph stage of *Cochliobolus sativus* (Phylum Ascomycotina, *order* Pleosporales). The sexual stage is extremely rare in nature (Tinline *et al*., 1988) and is not known to play any role in the epidemiology of the diseases. It produces multicelled, dark-colored conidia. The conidia are curved to straight, fusiform to broadly ellipsoidal and have a unipolar or bipolar germination (Fig. 3), hence the name *Bipolaris*. *B. sorokiniana* is mostly referred to as a necrotrophic fungus (kills and feeds on dead host cells). It does, however, also possess biotrophic properties (feeds on living host cells) that can be utilized during the initial infection of a host plant (Dehne & Oerke, 1985; Kumar *et al*. 2002). Hence, it is also referred to as a hemibiotrophic fungus.

Infection biology

In the early stages of a successful infection, conidia of *B. sorokiniana* adhere to the leaf surface and germinate within 6-16 hours (Agrios, 1997; Yadav, 1981). During germination, an extracellular matrix (ECM) is produced that attaches the germling to the surface to prevent the fungus from desiccation and also to serve as a reservoir for phytotoxins and plant cuticle degrading enzymes (Åkesson *et al*., 1995; Apoga & Jansson, 2000). Most soil-borne conidia are found in the top 15 cm of the soil (Piening, 1997). Germ tubes grow towards roots and root exudates of barley chemotropically to initiate primary infection on the coleoptiles or on the primary roots (Jansson *et al*., 1988). To be able to penetrate the host, the hyphae must adhere to the surface by producing appressoria (Clay *et al*., 1994). The tip of germ tubes can penetrate leaves directly or through stomata (Yadav, 1981). A combination of mechanical force and enzymes is normally required for effective host penetration (Pryce-Jones *et al*., 1999). Some barley cultivars produce papilla at attempted penetration sites of *B. sorokiniana*. It is seen as a swelling of the epidermal cell wall just below the appressoria and may lead to non-successful penetration by the pathogen (Kumar *et al*., 2001). Enzymes like cutinases and esterases known for their ability to degrade the cutin layer of the cuticle, and endopolygalacturonase and xylanase that degrades primary cell walls have been characterized from *B. sorokiniana* (Peltonen *et al*., 1994; Clay *et al*., 1997; Lin and Kolattukudy, 1980).

Successful penetration of barley leaves by *B. sorokiniana* occurs approximately after 16-19 hours and results in growth of intracellular hyphae in epidermal cells (Yadav, 1981; Kumar *et al*., 2001). The hyphae form an intercellular mycelium in invaded mesophyll leaf tissues, where cells at the infection site show early necrotic reactions and will die and collapse. In roots, hyphae predominantly colonize epidermal and outer cortex cells intracellularly and the inner cortex tissue intercellularly (Carlson *et al*., 1991a). Symptomless biotrophic development of *B. sorokiniana* can be seen in barley leaves under low light intensity where the fungal hyphae are capable of both penetrating turgid, living epidermal cells to produce haustoria-like infection structures and intensively colonize the intercellular space without causing visible damage to the tissue (Dehne & Oerke, 1985).

An initial infection of the leaves, nodes or internodes often results from air-borne conidia spread from wild grasses or from plant debris on the soil surface, carried by wind or splashing rain. Oval to round brown spots with chlorotic margins are formed. They are usually restricted in width by leaf veins and are later turned into elongated dark brown necrotic spots (Steffenson, 1997). Seedlings carrying seed-borne inocula are stunted. Roots and subcrowns that develop oval, dark brown lesions are usually infected by soil-borne inocula. The lesions form into long areas of necrotic brown to black tissue as the infection progress. When roots and crowns are only moderately infected, there are no immediately noticeable symptoms above ground.

Phytotoxin

Fungal toxins can be involved in all stages of infection from initial adhesion, penetration and spread through the host tissue to senescence of the infected tissue (Knoche & Duvick, 1987). The most active and abundant phytotoxin produced by *B. sorokiniana* is prehelminthosporol, a non-host-specific toxin (Carlson *et al*., 1991b).

Known functions of prehelminthosporol is its toxic effects on different membrane systems of the plant cell, causing increased leakage of ATP from barley roots (Liljeroth *et al*., 1994), inhibition of proton pumping, reduction of ATP hydrolysis and interference with calcium uptake mechanisms in barley root plasma membrane vesicles (Olbe *et al*., 1995). Leakage of electrolytes and nutrients from cells damaged by toxin would benefit the intercellular growth of the pathogen. Barley is, as mentioned earlier, able to form papilla by using a callose-producing enzyme, 1,3-beta-glucan synthase (Kauss, 1990). Helminthosporol, another toxin produced by *B. sorokiniana*, can inhibit the 1,3-β-glucan synthase activity in its host plant (Briquet *et al*., 1998). Therefore, helminthosporol may be involved in the penetration strategy of *B. sorokiniana* by suppression of papilla formation.

3. Aim and objectives

The aim of my PhD project was to study the spatial and temporal location of PR-1, PR-2, PR-3 and PR-5 in various tissues of barley and wheat, to better understand the role and possible function of PRs in defense against *B. sorokiniana*. The objectives of this thesis were to study:

- The spatial accumulation of PRs in barley and wheat in response to infection of root segments of different ages.
- The localization of PR-1 in leaves of barley in response to leaf infection on a tissue and subcellular level.
- The correlations between the timing in transcription, protein accumulation and localization of PRs, both locally and systemically, after infection of barley root or leaf tissues.

4. Methodological aspects

Plant material and fungal isolates

Two barley (*Hordeum vulgare*) cultivars, Alva and Golf, and the wheat (*Avena sativa*) cultivar Kadett were used. Both Alva and Golf have been used for feeding. Alva carries an ML-La allele resistant to isolate C15 of *Blumeria graminis* f. sp. *hordei* and reacts with HR upon infection. The two isolates of *B. sorokiniana* used, R002 and THA1, were obtained from R.W. Stack, Plant Pathology, N. Dakota State University, USA and J. Hetzler, Institut für Pflanzenpathologie und Pflanzenschutz, Göttingen-Wende, Germany, respectively. In a study by Almgren *et al*. (1999) R002 was found to be most aggressive on barley roots and THA1 most aggressive on barley leaves.

Inoculation methods

Several inoculation methods were used in the thesis. In paper I barley and wheat were pot grown and their root tips grew into a layer of inocula consisting of oat kernels colonized by *B. sorokiniana.* This was to study the accumulation of PRs in leaves and roots while mimicking natural conditions and, except from the inoculation, stress the plants as little as possible. Some seedlings were grown in paper rolls to facilitate inoculation of different parts of the root system with agar discs containing germinated spores of *B. sorokiniana*. This was performed to study if there was a difference in the PR protein accumulation depending on the age of the infected root segment and if a distal response is detectable in the root depending on infection site. Wheat and barley were also grown in glass boxes to study the impact of different infection sites on the local and distal accumulation in the root of PR-1 and PR-3 by immunolocalization.

Localization of PR-1 on a subcellular level was performed on barley leaves after spraying pot grown plants with an inoculum of *B. sorokiniana* (paper II).

In paper III and IV, a growth system was set up by using plastic plates with lids. Sterile, moist filter papers were placed on the plates upon which 10 barley seeds were placed in a row on the rim, where a 1 x 10 cm rectangular had been cut out. The plates were enclosed with lids covered with moist sterile filter paper on the inside, and placed in an upright position. The seedlings were inoculated on young root segments with agar discs containing germinated spores of *B. sorokiniana*, or with small droplets containing spores of *B. sorokiniana* on the first leaf (Fig. 4). This was performed to study differences in local and distal accumulation of PRs depending on the site of inoculation.

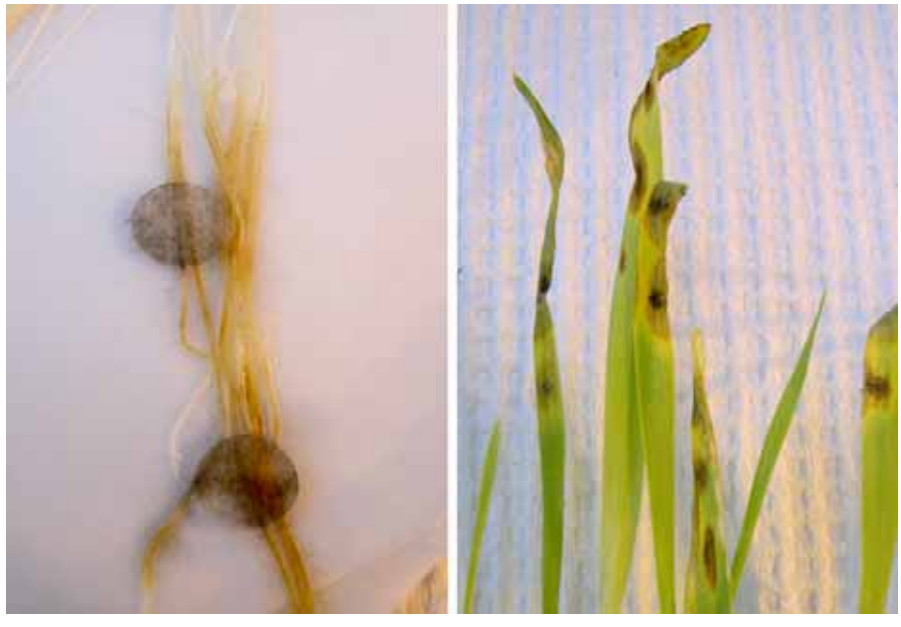

Figure 4. **Left**. *Young barley root segments inoculated with agar discs containing* B. sorokiniana*, 96 hai.* **Right**. *The first leaves of barley seedlings inoculated with small droplets containing spores of* B. sorokiniana*, 96 hai (© K. Santén).*

Isoelectric focusing (IEF)

IEF is a gel-based method to separate proteins in the native form according to their isoelectric points (pI) (Wilson & Walker, 2000). A small charge difference in a protein will be detected by IEF. Therefore the method is very useful for separating isoforms of PRs since they often differ only in pI value but not in size. PRs are often referred to as acidic or basic depending on their pI value.

Antibodies

Most methods used in this thesis are based on protein detection by antibodies. Antisera had earlier been raised in rabbits against all the PRs utilized in our studies. Rabbits injected (immunized) with a PR protein will recognize it as a foreign molecule, thus producing antibodies that will react particularly to this specific antigen (Wilson & Walker, 2000). The immune system produces a group of different antibodies with specific affinity to different parts (epitopes) on the antigen. Together they form a polyclonal population of antibodies as opposed to monoclonal antibodies that will only recognize one epitope on its antigen. I have only used polyclonal antibodies in my studies.

The effectiveness of immunochemical methods is to a large extent dependent on the quality of the antibodies used. Both their specificity and affinity towards the antigen will affect the results gained. It is also important to have a control serum, preferably a pre-immune serum, taken from the rabbit before it is immunized. With a pre-immune serum it is possible to exclude background labeling. In the case that pre-immune serum is not available it is possible to use non-immune serum, a pooled serum from several rabbits that have not been immunized. The primary antibodies can easily be detected by using secondary antibodies raised in another animal species against the constant region of the primary antibodies. The secondary antibodies are usually labeled with enzymes or colloidal gold to facilitate the visualization of the antibody. All PRs used as antigens for antibody production in this thesis originated from the extracellular washing fluid from barley leaves of cv. Alva, after infection by the powdery mildew fungus *Blumeria graminis* (Bryngelsoon & Greén, 1989; Bryngelsson *et al*., 1994).

Western blotting and enzyme-linked immunosorbent assay (ELISA)

Both western blotting and ELISA are based on the ability of a specific antibody to recognize its antigen in a mixture of other soluble proteins (Wilson & Walker, 2000). As the affinity of the antibodies varies, the methods are not suitable for absolute quantitative comparison between different proteins. *Western blotting* is used as a qualitative and/or semi-quantitative method. A mixture of extracted soluble proteins is loaded onto a polyacrylamide gel that will separate individual proteins by electrophoresis according to their size and charge. The protein-gel is blotted on to a membrane, where a specific protein can be detected separately as a band by using enzyme-linked secondary antibodies. *ELISA* is to gain quantitative information of a specific protein in a chosen tissue. It is based on a color change of the enzyme-linked secondary antibodies that is proportional to the amount of a specific antibody attached to its antigen in a mixed solution. The intensity of the color is measured by a spectrophotometer.

Immunodetection using light microscopy (LM) and transmission electron microscopy (TEM)

In immunomicroscopy antibodies were used to visualize the presence and location of specific PRs in tissue sections. There is always a risk that some proteins escape from tissue samples. Possible loss of antigens due to vacuum pumping and alteration of antigenicity due to fixation and embedding procedures can not be excluded. One of the most crucial steps in immunomicroscopy is the fixation where a combination of both good structural preservation and sustained antigenicity is important. Too much cross-linkage by the aldehydes used in fixation of the tissue may mask many epitopes. On the other hand, tissues may disintegrate without adequate fixation and antigens may simply be washed out. The increasing gradient of labeling in infected tissues indicated a satisfactory preservation of the antigenic sites. The secondary antibodies utilized in our microscopy studies were linked with electron-dense colloidal gold which is easily seen in TEM. For light microscopy the gold particles were enhanced by silver for visualization.

Northern blotting

To study if inoculation with *B. sorokiniana* resulted in activation of the genes corresponding to the PRs under study, we isolated RNA from several time points during the first four days after infection (dai). The presence of specific mRNA molecules in a tissue was visualized by northern blotting, a method where RNA is separated by gel electrophoresis according to size and transferred onto membranes. Thereafter specific mRNA molecules can be identified by hybridization of the membrane with sequence-specific gene probes made from complementary DNA (cDNA) clones of the sequence in search. The probe is radioactively labeled and the detected RNA appears as a distinct band when the membrane is exposed to an X-ray film. Concentration and purity of the RNA samples loaded onto the gel were determined with a NanoDrop (NanoDrop Technologies Ltd, USA) spectrophotometer. The quality of the RNA was further confirmed by agarose gel electrophoresis. The cDNA clones were kindly provided by Professor David Collinge, Royal Veterinary and Agricultural University, Copenhagen, Denmark.

5. Results and discussion

In the following, the accumulation and target sites of PRs in tissues local and distal to the infection site of *B. sorokiniana* will be described and discussed. Inoculations were performed on young and old segments of primary roots as well as on leaves. PR-1, PR-3 and PR-5 were studied to a greater extent than PR-2, which was only included in paper I.

5.1 Localization and accumulation in ground and dermal tissues

Leaves from leaf-infected seedlings

As seen on western blots from leaf-infected seedlings, it is clear that infection by *B. sorokiniana* results in increased accumulation of PR-1, PR-3 and PR-5 in barley leaves (Table 3). In paper III and IV, leaves exhibited different degrees of constitutive expression of all three PRs, possibly due to stress caused by the growth conditions.

Table 3. *Localization of PR-1, PR-3 and PR-5 on western blots based on results presented in papers II, III and IV to highlight changes in the spatial accumulation of the proteins in relation to infection site. Barley seedlings were analysed four to five days after inoculation with* B. sorokiniana

Treatment	PR-1		PR-3		PR-5	
	C	I	C	I	C	I
Leaves from leaf-infected	+	++	+	++	+	+++
Roots from leaf-infected	+	+	(+)	(+)	+	++
Leaves from root-infected	+	+	+	+	+	(+)
Roots from root-infected	+	++	(+)	+	+	+++

Note: The gradient utilized to compare the label intensity in the different tissues is restricted to the separate columns, i.e. *antibodies. C = non-infected seedlings; I = infected seedlings; (+)= low level; += intermediate level; ++= high level; +++= very high level*

Four to five days after inoculation (dai) both PR-1 and PR-5 were present in the cell walls of the epidermal layer (Table 4*)*. The innermost cell wall, facing the mesophyll, had a higher accumulation of PR-1 and to some extent also PR-5 whereas the outermost cell wall towards the cuticle was nearly free of label (Fig. 5a). PRs may have been located in the outermost cell wall at an earlier stage of infection and later been relocated to the innermost cell wall. At the time of sampling the fungus had already heavily invaded the ground tissue. Subcellularly, labeling of PR-1 was seen in papilla-like structures in the outermost part of epidermal cell walls 96 hai (paper II). These structures were most likely formed early during infection when the hyphae first tried to penetrate the epidermal layer. In contrast to PR-1 and PR-5, the PR-3 proteins were present at a very high density in the outermost wall of

epidermal cells, in infected as well as non-infected leaves (Fig. 5b; paper IV). This could explain the high constitutive level of PR-3 seen in western blots of leaves. Interestingly, western blots of wheat leaves did not exhibit the same constitutive level of PR-3 in non-infected leaves, although readily induced upon infection by *B. sorokiniana* (paper I). This may indicate that wheat possesses an isoform of PR-3 that our antibody has very low affinity for or that wheat has a differential accumulation pattern of PR-3 in the leaves compared to barley.

The basic PR-1 and PR-3 and the acidic PR-5 proteins appear to have target sites in common in infected leaf tissues, *i.e.* the host cell walls and the intercellular space of the ground tissue close to the infection site (Table 4). This confirms that PRs, whether they are basic or acidic, can be secreted extracellularly in barley leaves. In tobacco the intercellular localization coincides with acidic PRs, while most of the basic PRs are recovered in the vacuole (Neuhaus, 1999). All three PRs studied in infected barley leaves were also recovered intracellularly, mainly restricted to chloroplasts, and not the vacuole. Presence of PRs in chloroplasts could indicate that they have a role in protection of chloroplasts from alterations or damage. PR-1 is also present in chloroplasts of developing barley flowers (Liljeroth *et al.*, 2005). Both natural senescence and accumulation of PRs have been reported to occur early in barley seedlings (Tamás *et al.*, 1998; Liljeroth & Bryngelsson, 2001). Possible involvement of PRs in senescence has also been reported in dicotyledonous plants (Hanfrey *et al.*, 1996). Their observations suggest an involvement of PRs in the control of leaf senescence rather than direct defense against pathogens. However, the induction of senescence could also be seen as a response to a pathogen infection (Butt *et al.*, 1998) as *B. sorokiniana* is known to produce toxins that can induce leaf senescence (Hodges & Campbell, 1999).

The labeling intensity of host cell walls with PR-1, PR-3 and PR-5 varied depending on the proximity to the infection site, *i.e.* it decreased at a relatively close distance. All three PRs also had in common that less labeling was observed in host cell walls when in close contact with hyphae, indicating that PRs had been conveyed from the host cell wall to the hyphal cell wall (paper II, III and IV).

PR-1 is one of the most well studied PRs but its biochemical function is still not known. One proposed mode of action of PR-1 is to prevent or at least restrict fungal development (Benhamou, 1995). Presence of PR-1 in mechanical reinforcements such as host cell wall appositions and in junctions filled with electron-dense material between mesophyll cells support this theory (paper II). In our study PR-3 was hardly found in these structures (paper IV). PR-5 was not studied at a subcellular level.

Table 4. *Overview of immunolocalization by light microscopy of PR-1, PR-3 and PR-5 in barley leaves in relation to infection site four to five days after inoculation with* B. sorokiniana*. The data are based on observations in paper II, III and IV*

Protein and treatment / Leaf tissue	PR-1			PR-3			PR-5		
	Lc	Ll	Lr	Lc	Ll	Lr	Lc	Ll	Lr
Dermal									
Outermost cell wall	+/-	+/-	+/-	++++	++++	++++	+/-	+/-	+/-
Innermost cell wall	-	+	-	+	+	+	-	+/-	-
Ground									
Mesophyll cell wall	-	+	-	-	+	-	-	+	-
Intercellular space	-	+	-	-	+	-	-	+	-
Mestome sheath	+/-	+	+/-	-	-	-	+	++	++
Vascular									
Xylem	+	++	+	-	-	-	+	++	++
Phloem	+/-	++	+	+	+++	++	-	++	+

Note: The gradient utilized to compare the label intensity in the different tissues is restricted to the separate columns, i.e*. antibodies. Lc = Leaves from non-infected; Lr = Leaves from root-infected; Ll = leaves from leaf-infected. - = nearly free of label; +/- = varying label intensity from nearly free to present; + = label; ++ = intense label; +++ = very intense label;++++ = extremely intense label.*

Roots from leaf-infected seedlings

After leaf-infection, no increased accumulation of PR-1 and PR-5 was detected in western blots on the whole root system or by immunolocalization in young root segments (paper III). However, immunolocalization of PR-3 did show some changes (paper IV, Table 5). In the cortex of young roots from leaf-infected plants, PR-3 was mainly found in the inner cortex compared to non-infected roots where PR-3 was recovered in the outer part of cortex (paper IV). One reason could be that the change in localization is a distal response to the pathogen attack in aerial parts, in case of further invasion via the vascular system. The low constitutive level on western blots of barley roots labeled with PR-3 in paper III when compared to paper I could be due to differences in the respective growth systems, the titer or batch of antisera used in the analyses.

Table 5. *Overview of immunolocalization by light microscopy of PR-1, PR-3 and PR-5 in barley roots in relation to infection site at four to five days after inoculation* with B. sorokiniana. *The data are based on observations in paper II, III and IV*

Protein and Treatment / Root tissue	PR-1			PR-3			PR-5		
	Rc	Rr	Rl	Rc	Rr	Rl	Rc	Rr	Rl
Dermal									
Outermost cell wall	++	++	++	-	-	-	+	+	+
Innermost cell wall	-	-	-	-	+/-	-	-	+/-	-
Ground									
Outer cortex									
cell walls	+/-	+	+/-	++	+/-	-	-	+/-	-
intercellular space	-	-	-	-	+/-	-	-	+/-	-
Inner cortex									
cell walls	+/-	+	+/-	+/-	+/-	++	-	+/-	-
intercellular space	-	-	-	-	+/-	++	-	+/-	-
Endodermis	+	/	+	-	/	-	++	/	++
Vascular									
Xylem	+	++	+	-	+/-	-	++	++	++
Phloem	+	+	+	+	+++	++	+	+	+

Note: The gradient utilized to compare the label intensity in the different tissues is restricted to the separate columns, i.e. *antibodies. Rc = roots from non-infected; Rl = roots from leaf-infected; Rr = roots from root-infected. - = nearly free of label; +/- = varying label intensity from nearly free to present; + = label; ++ = intense label; +++ = very intense label; / = tissue not present.*

Seedlings with root-infection in young or old root segments

As seen with ELISA, inoculation with *B. sorokiniana* close to the root tip resulted in an increase of PR-1, PR-2, PR-3 as well as PR-5 in older root segments (paper I). Some of these results were confirmed by immunolocalization, where an increase in PR-1 accumulation was seen in the stele and the inner cortex of older root segments after infection of young roots. However, it was not possible to detect an increase of PR-3 distally in older root segments although a high constitutive level in the cortex of older barley roots was found. In wheat, the localization study did not show any increase neither in PR-1 nor PR-3 in older root segments after infection close to the root tip.

In the reciprocal experiment, where old root segments were inoculated, no significant increase in PR protein accumulation was found in the root tips. These results were also confirmed by immunolocalization of PR-1 and PR-3, although a small increase of PR-1 and PR-3 in the stele of barley root tips was observed. A relatively high constitutive level of PR-1 and PR-3 was found in the cortex of young

root segments from both barley and wheat. These differences in distal induction, depending on the site of infection, may relate to the fact that the older root segments could suffer from cortical cell death (Deacon, 1987; Liljeroth & Bryngelsson, 2001). Programmed cell death is known to start at an early stage in the cortex of cereal roots (Liljeroth, 1995). An early decrease in vital root cells may affect the ability of older root segments to recognize a pathogen and respond to a subsequent infection effectively.

Roots from root-infected seedlings

The local induction of PRs in roots was studied using IEF, ELISA, western blotting and immunomicroscopy. The accumulation of PR-1, PR-3 and PR-5 increased within 24 hai in roots. PR-3 had a less pronounced increase, as seen on western blots, although ELISA analysis revealed a significant increase of PR-3 in extracts from barley roots infected close to the root tip (paper I). The level of PR-1 found in endodermis differed in paper I and paper III (Table 5), most likely due to the differences in growth conditions and infection pressure. The two barley cultivars Alva and Golf may also differ in their response to infection. In paper III both PR-1 and PR-5 were present in the endodermis but did not show a strong increase after infection. The cortex had thickened cell walls but the labeling was not dense either with PR-1, PR-3 or PR-5. However, invading hyphae were densely labeled. Interestingly, the epidermal layer of roots was densely labeled with PR-1 and PR-5 but not with PR-3, which is opposite to the infected leaves where a strong label was seen with PR-3 and scarcely at all with PR-1 and PR-5.

Leaves from root-infected seedlings

In the first study, signs of PR-1, PR-3 and PR-5 induction in leaves after infection of root tips in barley was found using IEF and western blotting. This was, however, not the case in western blottings from wheat seedlings where no accumulation at all was detected of PR-1, PR-3 and PR-5 in leaves from root-infected plants.

There was no clear difference in the amount of accumulation of PR-1 or PR-3 proteins in leaves from root-infected compared to the level in non-infected seedlings at 96 hai in western blots in paper III and IV (Table 3). However, a decrease in PR-5 was detected within 12 hai and will be discussed in the next chapter 5.2. Immunolocalization of PR-1, PR-3 and PR-5 revealed no increase or novel localization sites for PRs in the epidermal or ground tissue in leaves from root-infected barley seedlings.

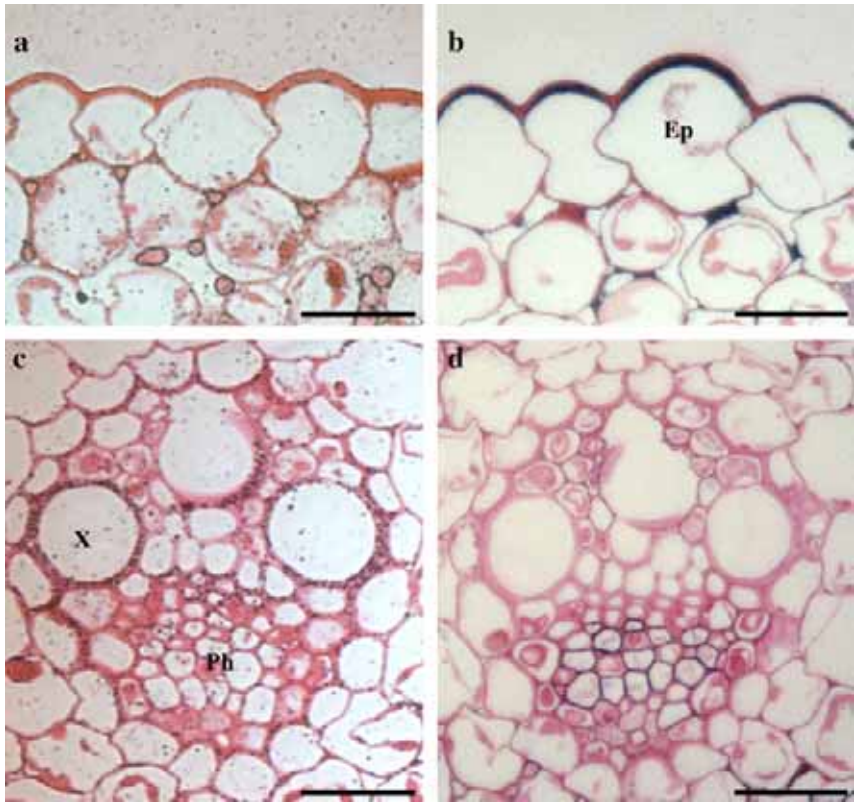

Figure 5. *Immunolocalization in leaves infected by* B. sorokiniana *using light microscopy. The outermost cell wall of epidermis labeled with antibody against* **a**) *PR-5;* **b**) *PR-3 and the vascular bundle with antibody against* **c**) *PR-5;* **d**) *PR-3. Labeling is seen as black dots or lines against the red Safranin background. Ep=epidermis; X=xylem; Ph=phloem. Bar 50 μm.*

5.2 Vascular localization and possible transport

Plants have evolved a vascular tissue with two long-distance transport systems, the phloem and the xylem. The xylem transports water and inorganic nutrients from the roots, while the phloem mainly transports organic compounds produced in the leaves to the roots or developing seeds. The vascular system is also thought to coordinate growth, development, repair, and defense reactions in plants (Buhtz *et al*., 2004, Kehr *et al*., 2005). The venal network of the leaf is the major site for assimilate loading and export (Turgeon, 1989). The vascular tissue is also most likely involved in the accumulation and/or movement of defense-related molecules.

Somewhat different results on the localization of PR-1 and PR-3 were found in paper I compared to papers III and IV. The differences may be due to the different growth systems, infection pressure and cultivars utilized. Both PR-1 and PR-5 were localized in the vascular tissue: the highest density was seen on the secondary cell walls of the xylem vessels and the tracheary elements of leaves, on the inner cell wall of the mestome sheath surrounding the vascular bundles of leaves (Fig. 5c) and

on the endodermis of roots (paper III, Table 4 and 5). Some labeling was also recovered in the cell walls of sieve tube elements in the phloem. In leaf- and root-infected seedlings, xylem and phloem of the minor veins as well as the intercellular space surrounding the minor veins had a dense labeling. The presence of PR-1 and PR-5 is either the result of protein synthesis in the vascular tissue or transport of protein into it. PR-1 and PR-5 could function as defense molecules on site or be in transit, loaded into and/or out of the xylem and phloem sieve tubes for further transport. Both PR-1 and PR-5 were constantly present in the vascular tissues in this study (paper III). In comparison, PR-1, PR-2, PR-3 (class I and IV) and PR-5 have been detected in the xylem sap of healthy *B. napus* plants (Kehr *et al*., 2005) and in the guttation fluid from non-infected barley seedlings (Grunwald *et al*., 2003). They observed that PR-5, among others, were not only constitutively present in the guttation fluid but also enriched in the fluid relative to the total proteins of a non-infected plant. I found labeling of PR-5 between adjacent phloem cells in the leaves from root-infected seedlings indicating a movement of PR-5 through the plasmodesmata between companion cells and sieve tubes (paper III). Bortolotti *et al*. (2005) demonstrated transport of a PR-1-like protein from companion cells into the sieve elements via plasmodesmata, resulting in a long-distance transport of PR-1 in the maize phloem. Western analysis in paper III indicated a decrease of PR-5 at 12 hai in the leaves of root-infected seedlings. This result may indicate a systemic translocation of PR-5 from healthy leaves to distal tissues that will be discussed in chapter 5.4.

In vascular tissue, PR-3 was almost exclusively localized to the phloem of both leaves and roots and was scarcely found in the mestome sheath or in endodermis (Fig. 5d; paper IV). In leaves, the phloem labeling had a tendency to increase also in root-infected seedlings. However, such an increase was not detectable on western blots. In roots the increase in labeling was most prominent in the infected roots, although roots from leaf-infected seedlings had a tendency to stronger labeling in the parenchyma cells of the stele. No PR-3 was detected in plasmodesmata of either leaves or roots. Even so, PR-3 appeared to increase distally in roots in response to leaf infection. Whether this was due to actual transport of PR-3 from leaves to the roots or due to transmission of signals and subsequent *in situ* synthesis of PR-3 could not be clarified with the methods used. The increase observed in roots was not confirmed by the corresponding western blot. The phloem tissue in leaves showed a slightly increased labeling in root-infected seedlings, but increased accumulation was not observed in the mesophyll of leaves. Interestingly, PR-3 was spread over the primary cell wall of the sieve tubes in leaves, whereas in roots PR-3 was localized in the middle lamella between sieve tubes and between sieve tubes and companion cells. PR-3 present in the middle lamella may elicit further defense responses and/or damage the hyphal tips emerging in the lamella. This could activate a proposed signal perception system in the phloem and induce defense proteins systemically (Walz *et al*., 2004).

5.3 Pathogen *in planta*

The primary hyphae of *B. sorokiniana* are composed of a layered cell wall structure resembling the wall structure of *F. oxysporum*. The outer cell wall layer of *F. oxysporum* has an electron-dense nature, containing cell wall glycoproteins (CWP), and a fibrous chitin-containing polysaccharide-rich inner layer that exhibits a more electron-transparent appearance (Schoeffelmeer *et al*., 1999). As reported in paper II, *B. sorokiniana* may have both thick-walled, large primary hyphae and smaller secondary hyphae, seemingly devoid of cytoplasm, with thinner cell walls.

The cell walls of *B. sorokiniana* primary hyphae were labeled with PR-1, PR-3 and PR-5 (paper II, III and IV). An extracellular matrix (ECM), that seemed to derive from hyphae invading the intercellular space of the mesophyll in leaves, was heavily labeled with all three PR antibodies. In our study, PR-1 was localized to the outer cell wall layer and PR-3 to the inner part of the fungal cell wall of primary hyphae (paper II and IV). The outer cell wall surface of a pathogen may contain specific components that control the passage over the cell wall by either facilitating or hindering the entrance (Veronese *et al*., 2003). We could see a clustered labeling of PR-1 in the cytoplasm of primary hyphae, both in seemingly healthy and clearly affected hyphae (paper II). It was not possible to determine whether the PR-1 found carried out antifungal activities or if it was caught in the hyphal cell wall by a fungal defense barrier. Due to its relatively small size it is also possible that PR-1 has entered passively through the nutrient uptake by the fungus. However, PR-3 was not detected intracellularly in the fungal hyphae. The localization of PR-3 on the more electron-transparent part of the fungal cell wall (paper IV) correlates well with studies on *F. oxysporum* (Arlorio *et al*., 1992; Benhamou *et al*., 1990). This localization of PR-3 is not surprising as it is a chitinase and is believed to hydrolyze accessible chitin that is present in most fungal cell walls. Released chitin fragments may thereafter act as elicitors and provoke further plant defense reactions (Boller *et al*., 1983).

The cell walls of secondary hyphae were nearly free of PR-1 labeling. From a fungal point of view, the advantages of dimorphism in the infection hyphae can be several. Large initial primary hyphae with a thick cell wall may both protect the hyphae from different host defense mechanisms and minimize the area of contact with the host cell wall. Small secondary hyphae with thin cell walls are more efficient in nutrient uptake and transport of toxins and cell wall degrading enzymes (Perfect *et al*., 2001). It would have been interesting to study the more specific location of PR-5 on the cell wall of *B. sorokiniana* since it has been reported that a specific CWP present in the outer cell wall of yeast can promote uptake of PR-5 across the fungal cell wall (Ibeas *et al*., 2000). Another yeast strain, however, was resistant to PR-5 due to its ability to block its passage at the cell surface by utilizing another CWP containing internal repeats (PIR) (Ibeas *et al*., 2001). Overexpression of a PIR protein in *F. oxysporum* did increase the virulence of the pathogen *in planta* and its resistance to tobacco PR-5 (Narasimhan *et al*., 2003). Although our observations do not bring conclusive evidence of a possible antifungal activity of PR-1, PR-3 and PR-5 to restrict fungal colonization, their localization sites *in situ* indicate that they interact with the fungal cell wall of *B. sorokiniana.*

In leaves, hyphae of *B. sorokiniana* were restricted mainly to the dermal and ground tissues. They were not recovered in the vascular tissue of leaves, even when the surrounding tissue was completely necrotic. In roots, hyphae heavily invaded both xylem and phloem tissues. Since the mesophyll cells in barley are richer in carbohydrates than the cortex cells of roots they may be the primary targets. The stele, transporting both carbohydrates and mineral-containing water, could be a preferable target for the pathogen in the root. The more aggressive invasion of the roots may also be due to the fungal isolate, R002, which is particularly aggressive in roots (Almgren *et al.*, 1999). The inoculated roots were very young and the protective endodermal cell layer had not developed fully. Additionally, the roots were, as opposed to the leaves, infected with sugar-containing agar discs with *B. sorokiniana* conidia. Hyphae invading roots therefore had an extra energy source.

5.4 The biphasic accumulation pattern in leaves

My studies demonstrate differential gene expression in barley leaves during the early phase of infection, before any visible symptoms are apparent in the tissues (paper III and IV). Necrosis of barley leaves and roots inoculated with *B. sorokiniana* appeared after approximately 36 hours. However, the northern blot analysis revealed a biphasic accumulation of PR-1, PR-3 and PR-5 mRNA in leaves from both leaf- and root-infected seedlings before these visible symptoms. mRNA of all three PRs accumulated within three hai. This first peak did not sustain, as the level of mRNA was as low as in non-infected controls 6-9 hai. Thereafter all three mRNAs started to increase again until at least 48 to 96 hai. The corresponding western blot analysis of the leaves from leaf- and root-infected seedlings showed a similar biphasic pattern in PR-1 and PR-5 protein accumulation, suggesting that the transcripts present at three hai had been translated into PR-1 and PR-5 proteins. It was not possible to detect changes in the accumulation of PR-3 protein at three hai, which may be due to the fact that the high level of constitutively expressed PR-3 made it difficult to detect small changes in protein accumulation. In leaves from leaf-infected seedlings the second steady increase in PR-1, PR-3 and PR-5 mRNA resulted in a durable accumulation of the corresponding proteins.

Interestingly, a difference was noticeable in the accumulation pattern of the corresponding proteins in leaves from root-infected seedlings. The second accumulation of PR-1 and PR-3 mRNA did not lead to any higher amount of PR-1 or PR-3 protein, and the PR-5 protein did in fact decrease over time. Neither leaves nor roots from non-infected seedlings exhibited a similar biphasic transcription pattern.

This type of early host recognition and induced biphasic transcript accumulation within hours after a fungal infection has been reported previously in barley seedlings (Clark *et al.*, 1993; Gregersen *et al.*, 1997; Christensen *et al.*, 2002). A biphasic pattern has been observed in the oxidative burst in plants under pathogen attack (Baker & Orlandi, 1995). The earliest reaction detectable after pathogen recognition is the formation of AOS (Garcia-Brugger *et al.*, 2006) in two distinct phases (Allan & Fluhr, 1997; Lamb & Dixon, 1997). The first burst, relatively weak and rapid, occurs within minutes and appears to involve an elicitor-receptor interaction,

whereas the second massive and sustained burst occurs within a few hours and correlates with HR (Allan & Fluhr, 1997). Using the same PR-1 cDNA clone as we have used, a correlation between PR-1 mRNA accumulation and H_2O_2 in barley leaves after infection with *B. sorokiniana* has been reported (Schultheiss *et al*., 2003). The biphasic induction pattern of PR-1, PR-3 and PR-5 mRNA in leaves from leaf-infected seedlings may indicate that the induction of PRs is sensitive to changes in AOS. My experiments revealed that the same temporal expression pattern appears in leaves after root infection with *B. sorokiniana*. This suggests that the perception of a pathogen on the host surface of the roots provokes a detectable response in the transcription of PRs in the leaves. Felle and coworkers (2005 & 2007) showed that barley plants utilize electrical signals systemically, *i.e*. action potentials (APs) for intercellular long distance signaling from root to leaf and from leaf to leaf when triggered by mild salt stress. APs are accompanied by temporary cytoplasmic decrease in pH, a change interpreted as a prerequisite for defense-related gene activation (Felle & Zimmermann, 2007; He *et al*., 1998). The APs also, within minutes, exhibit a biphasic pattern although the voltage change is very fast (Felle & Zimmermann, 2007). The biphasic pattern in mRNA accumulation detected in leaves from root-infected seedlings may be the result of a distal root-to-shoot signal and as no invading pathogen is recognized on the leaf surface, no second phase of AOS is initiated in the leaves. The lack of a clear increase of PR-1 and PR-3 proteins after 3 hai, as in leaves from leaf-infected seedlings, is consistent with this hypothesis (paper III and IV).

The decrease in protein level of PR-5 in leaves after root-infection indicates a possible systemic translocation of PR-5 from healthy leaves to distal tissues. This is supported by Narvaéz-Vásquez (1995) who found that translocation of polypeptides, via phloem throughout the entire plant, can take place within a couple of hours after wounding. The dense labeling of PR-5 between adjacent phloem cells, indicating movement of PR-5 in plasmodesmata between companion cells and sieve tubes, also suggests transport in the phloem. There was no detectable decrease in the level of PR-5 in roots from leaf-infected seedlings, which indicates that a reverse translocation of proteins from roots towards distal infected leaf tissues was not occurring. However, the constitutive presence of PR-1 and PR-5 in the xylem tissue correlates well with the detection of PRs in the guttation fluid from leaves of non-infected barley seedlings reported by Grunwald *et al*., (2003).

6. Conclusions and future prospects

Conclusions that can be drawn from this thesis are:

- The basic barley PR-1, PR-2 and PR-3 and the acidic PR-5 proteins accumulate in both leaves and roots of barley and wheat seedlings when inoculated in the respective tissue with the hemibiotrophic fungus *Bipolaris sorokiniana*.
- PR-1, PR-3 and PR-5 mRNA accumulate in a biphasic pattern in leaves from both leaf- and root-infected seedlings. This pattern is not detectable in roots.
- The accumulation of PRs in roots is dependent on whether young or old root segments are infected. A stronger response, both locally and distantly, was found when young root segments were inoculated in comparison to old roots.
- PR-1 and PR-5 are constitutively present in the vascular tissues and also in the root epidermis. In response to infection the proteins are induced in the ground tissues with an additional increase of the PRs in the vascular tissue.
- The basic barley PR-3 protein displays a tissue-specific difference between leaves and roots. PR-3 is constitutively present in leaves, whereas roots have a low constitutive level. The difference in the constitutive level originates from the dense labeling of the cell walls of the epidermal layer exclusively in the leaves.
- The basic PR-3 protein is mainly restricted to the phloem of the vascular tissue of young infected roots, whereas PR-1 and especially PR-5 are recovered in both xylem and phloem tissues.
- PR-1, PR-3 and PR-5 are recovered over the cell walls of inter- and intracellular hyphae of *B. sorokiniana* indicating that they may influence fungal growth.
- The basic PR-1 was not found in vacuoles of infected barley leaf seedlings as shown with other basic PRs in dicotyledonous plants. However, chloroplasts were labeled with the PR-1 antibodies.

In this thesis I have primarily focused on where different PRs are localized in infected and non-infected tissues in a monocotyledonous plant species to broaden the understanding of their possible function in plant defense. It would be interesting to study in more detail where and when different PRs are transcribed to better understand how plants utilize and coordinate their defense mechanisms.

Based on the sequence data for different isoforms it would be possible to produce isoform-specific antibodies and to design specific primers. It has been shown that some isoforms of PRs possess antifungal activity. It would be interesting to study where different isoforms are localized and how they are coordinated in plant

defense. A tissue-, time- or pathogen-specific expression and localization study on different isoforms could shed more light on their specific functions.

Since not many microscopy studies have been reported on PR-5 and as it is considered to posses antifungal properties it would be interesting to locate PR-5 on a subcellular level to gain more insights into its specific functions.

Presence of PRs in xylem and phloem indicates that they may be transported long distances in the vascular tissue. A study of possible transport of PRs to and/or from infected ground tissue via the vascular system to other parts of the plant and in which tissue the PRs for transport are transcribed would tell us more about how plants utilize their vascular system in defense.

It would be valuable to know if the pure barley PRs have antifungal activity against *B. sorokiniana*, separately or in various combinations and in more detail to see how the fungus is affected. Since *B. sorokiniana* secretes the toxin prehelminthosporol it would be of interest to see where it is recovered in the tissue, and if it affects the host response in terms of localization and accumulation of different PRs.

There are cultivar differences in barley resistance towards *B. sorokiniana*. These genetic differences play an important role in the early crucial recognition of a pathogen and the subsequent timing of defense responses in a plant. In our studies changes in mRNA accumulation occurred within the first six hai. It would be interesting to compare susceptible and resistant cultivars in the early stages of infection to see where and when transcribed mRNA and the subsequent PRs are located in correlation with adhesion, penetration attempt and HR.

Abbreviations

AFP	antifreeze protein
AOS	active oxygen species
AP	action potential
CWP	cell wall glycoprotein
dai	days after inoculation
ECM	extracellular matrix
hai	hours after inoculation
HR	hypersensitivity response
HRGP	hydroxyproline-rich glycoproteins
JA	jasmonic acid
LM	light microscopy
pI	isoelectric point
PIR	protein containing internal repeat
PRm	pathogenesis-related PR-1-like protein expressed in maize
PRs	pathogenesis-related proteins
SAR	systemic acquired resistance
SA	salicylic acid
TEM	transmission electron microscopy
TL	thaumatin-like

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Acknowledgements

First of all I owe special thanks to six people who have had particular importance for my fulfilling of this work:

Prof. Tomas Bryngelsson, my main supervisor. Thank you Tomas, for given me the opportunity to do my PhD-studies in your research group. You have always given me full freedom to work in my own way, something I value highly.

Doc. Erland Liljeroth, my second supervisor. Thank you Erland for all your good advice and valuble support.

Dr. Salla Marttila, my third supervisor. Thank you Salla, for everything. You have always done your best to help, encourage and inspire me, in ways I will never forget. Without your knowledge and support this thesis would not have been completed.

Prof. Waheeb Heneen, my fourth supervisor. Thank you Waheeb, for always listen and encourage me in my work and life.

Mr. Donald Ribbentjärn, my personal coach. Thank you Donald for your hard work and your engagement in my goal and me. You manage to make me put important things in new perspectives, not an easy task.

Mrs Iris Santén, my mother. Thank you for always being there, helping me, supporting me and making my life and dreams possible.

To colleges and friends
This has been a long hard, marvelous journey for me. On this journey I have had the privilege to meet and work with so many wonderful people. You have helped me through laughter, tears and hard work in so many ways, so many times. I owe you all my deepest most hearty **Thank you**.

My last acknowledgment is the most important. My dear **Stefan** and our wonderful children **Oskar** and **Alice**, I would be nothing without you