University of Alberta

A proteome-level analysis of the canola/*Sclerotinia sclerotiorum* interaction and sclerotial development

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Plant Science

Department of Agricultural, Food and Nutritional Science

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Fall 2010

Edmonton, Alberta

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Abstract

The fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary is capable of infecting over 400 plant species including canola (*Brassica napus* L.). The fungus secretes oxalic acid (OA), which plays an important role in infection and disease progression. An analysis of proteome-level changes associated with infection of susceptible canola leaves by *S. sclerotiorum* revealed significant changes in the abundance of 32 proteins, including proteins involved in photosynthesis and metabolism, hormone signaling, and antioxidant defense. A similar subset of 37 proteins was affected when leaves were treated with OA alone; this compound also caused a reduction in the activities of a number of antioxidant enzymes, suggesting an OA-mediated suppression of the oxidative burst. To further understand the mechanisms of pathogenesis, the role of Sssp, a predicted secreted protein from *S. sclerotiorum*, was targeted for analysis. Mutant strains of *S. sclerotiorum* were generated by disruption of the *Sssp* gene and characterized for virulence on canola. Based on the extent of symptom development, the virulence of the Sssp-disrupted mutants was significantly reduced relative to the wild-type, indicating that Sssp may play a role in the infection process. Finally, the development of sclerotia, long-term survival structures that serve as a primary source of inoculum for the fungus, was examined. A total of 88 proteins were found to exhibit temporal changes in abundance during sclerotium formation and maturation, including proteins involved in the regulation of melanogenesis. A total of 56 proteins were also identified in the sclerotial exudates, providing a basis for future studies.

Collectively, the studies described in this dissertation represent the most comprehensive proteome-level analysis of the canola/*S. sclerotiorum* interaction and sclerotial development, and could contribute to the development of novel strategies for the management of *S. sclerotiorum*.

Acknowledgements

I sincerely express my appreciation to Drs. Nat Kav and Stephen Strelkov for their valuable guidance and support during the pursuit of my PhD degree at the University of Alberta in Canada. I am also grateful to Dr. Michael Deyholos for providing his suggestions as part of my Supervisory Committee. I would like to thank Drs. Lloyd Dosdall, Chris Willenborg, and Christof Rampitsch for serving as members of my Examination Committee.

The completion of this thesis was made possible with the assistance of a number of people. The technical support provided by staff at the Institute for Biomolecular Design (Department of Biochemistry, University of Alberta), the Molecular Biology Service Unit, as well as the Microscopy Unit (Department of Biological Science, University of Alberta) is greatly appreciated. The assistance of all members of Dr. Kav's and Dr. Strelkov's laboratories, as well as Bruce Alexander, Laura Smith, and Jody Forslund, is also appreciated. Funding from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Alberta Agriculture Research Institute (AARI), the A.W. Henry Endowment Fund, the China Scholarship Council (CSC), and the Department of Agricultural, Food and Nutritional Science (AFNS) is gratefully acknowledged.

Finally, I sincerely express my admiration for the constant love from my parents (Mrs. Surong Tian and Mr. Baoquan Liang) and for the obliging support of everyone in my life.

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List of abbreviations

2-DE: 2-dimensional gel electrophoresis

BSA: bovine serum albumin

CAT: catalase

CP: cerato-platanin

CWDE: cell wall degrading enzyme

DTT: dithiothreitol

ESI: electrospray ionization

EST: expressed sequence tag

FA: formic acid

FAOSTAT: Food and Agriculture Organization statistics (United Nations)

FBPase: fructose bisphosphate aldolase

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

HPH: hygromycin phosphotransferase

HPI: hours postinoculation

IEF: isoelectric focusing

IPG: immobilized pH gradient

JR: JA-responsive

MALDI: matrix assisted laser desorption/ionization

MAT: methionine adenosyltransferase

MS: mass spectrometry

MWCO: molecular weight cut-off

NOX: NADPH oxidase;

NPP1: necrosis inducing protein

OA: Oxalic acid

OXO: oxalic acid oxidase

PCR: polymerase chain reaction

PDA: potato dextrose agar

POX: peroxidase

PR: pathogenesis related

ROS: reactive oxygen species

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SOD: superoxide dismutase

Ssp: development-specific protein

TCA: trichloroacetic acid

TOF: time of flight

TPI: triosephosphate isomerase

UROD: uroporphyrinogen decarboxylase

1. General Introduction

1.1. Plant diseases

Plants contribute to the quality of the environment on our planet in many ways, including by providing the oxygen that we breathe and the food that all animals, including humans, consume either directly or indirectly. Normal or healthy plants can thrive when provided with sufficient nutrients and proper environmental conditions (e.g., moisture, light, and temperature) for growth. However, one of the major limitations affecting plant growth and productivity is the diseases that affect them. Plant diseases can cause significant losses, including reductions in the quantity and quality of plant products, as well as limitations in the kinds of plants that can grow in certain areas. Ultimately, these losses can result in reduced availability of food, feed and fiber, as well as major financial losses to farmers and society as a whole (Agrios, 1997).

Plant diseases can be classified according to the symptoms that they cause (e.g., rots, mildews, wilts, spots, blights, rusts), the organs they affect (e.g., root diseases, stem diseases, foliage diseases), and/or the type of host that they infect (e.g., field crop diseases, vegetable diseases, turf diseases). A broader classification, however, is based on whether a disease is caused by pathogenic organisms, i.e. biotic factors (e.g., fungi, bacteria, viruses, nematodes, parasitic higher plants), or environmental or abiotic factors (e.g., temperature extremes, limiting water conditions including drought and salinity).

The Great Irish Potato Famine in the 1840s greatly stimulated interest in the cause and control of plant diseases, and marked the birth of plant pathology as its

own discipline (Agrios, 1997). Since then, there have been great advances in understanding of plant diseases and their development. While initially focused mainly on descriptions of disease, plant pathology has become a much broader discipline. Research activities within the field are aimed at the development of chemical and alternative disease control protocols, enhanced understanding of the mechanisms by which pathogens cause disease, genetic inheritance of resistance and pathogenicity, as well as the epidemiology of plant diseases. In recent years, advances in molecular plant pathology have contributed to rapid strides in understanding various aspects of infection by a pathogen and disease progression in the host, including the identification of genes involved in mediating plant responses to the pathogen (e.g., resistance or tolerance). In this dissertation, a proteomics-based approach was used to the study canola/*Sclerotinia sclerotiorum* pathosystem.

1.2. *Brassica napus* (canola)

Four polymorphic plant species in the *Brassica* genus are cultivated worldwide, including *B. juncea* L., *B. napus* L., *B. oleracea* L., and *B. rapa* L.. The high chromosome number species *B. napus* (genome, AACC; 2n=38), *B. juncea* (genome, AABB; 2n=36) and *B. carinata* A. Braun (genome, BBCC; 2n=34) are derived from the low chromosome number species *B. rapa* (genome, AA; 2n=20), *B. nigra* (genome, BB; 2n=16) and *B. oleracea* (genome, CC; 2n=18) (Nagaharu, 1935). Canola was developed in Canada in the 1970s, from *B. napus* and *B. rapa*, as an edible oilseed crop having low erucic acid and

glucosinolate content in the seeds (Stefansson, 1983). Global production of rapeseed, including canola, was approximately 49 million metric tones (MMT) in 2007, with the major producers being Canada, China and India, among other countries (FAOSTAT data 2007).

Several major diseases can affect the yield and quality of canola, including damping-off and seedling blight caused by *Rhizoctonia solani* J.G. Kühn, *Fusarium* and/or *Pythium* species; clubroot caused by *Plasmodiophora brassicae* Woronin; Alternaria black spot caused by *Alternaria brassicae* (Berk.) Sacc., *A. raphani* J.W. Groves & Skolko, and *A. alternata* (Fr.) Keissl.; blackleg caused by *Leptosphaeria maculans* (Desm.) Ces. & de Not.; and Sclerotinia stem rot, caused by *Sslerotinia sclerotiorum* (Lib.) de Bary , which is the focus of this dissertation.

1.3. *Sclerotinia sclerotiorum* and stem rot

Sclerotinia stem rot is one of the many diseases caused by *S. sclerotiorum*. This fungal pathogen causes extensive yield losses in many field and horticultural crops (Purdy, 1979; Steadman, 1983; Boland and Hall, 1994). Stem rot can be difficult and cost-ineffective to manage using routine fungicide applications because severe and typical outbreaks of the disease take place randomly in both time and space (Bom and Boland, 2000).

1.3.1. Symptoms

Stem rot symptoms first appear near the late flowering stage of many crops. Small, watery, light brown spots develop on infected petals, which later enlarge and extend to the entire petal; the whole flower eventually turns dark brown and

drops off the plant (Agrios, 1997). Infected leaves usually develop water-soaked lesions that extend rapidly down the petiole or leaf axil and into the stem. These lesions usually turn necrotic, and subsequently a white-cottony mycelium appears, a characteristic sign of plant infection by *S. sclerotiorum* (Bolton et al., 2006). The development of numerous black sclerotia, which are hard overwintering structures produced by the fungus, is the most obvious sign of the pathogen in or on the soil, infected plant tissue (especially stems) and crop debris. Sclerotia will often be found embedded in the cottony white mycelium inside the infected stems. They may also be found on the outside of the stems, especially under damp conditions (Bolton et al., 2006).

1.3.2. Host range

Sclerotinia sclerotiorum is a necrotrophic fungus, which depends upon dead organic matter as its food source (Bateman, 1964). Many plants, including 75 families, 278 genera, and more than 400 species, are reported to be susceptible to infection by this pathogen (Boland and Hall, 1994). In Canada, more than 100 species of plants serve as hosts, including agriculturally important crops such as sunflower, dry bean, canola, pea, carrot, celery, and soybean. However, members of the cereal or grass family (Poaceae), including small grains and corn, are usually considered to be immune to infection by *S. sclerotiorum.* A survey of the literature revealed over sixty names used to refer to diseases caused by *S. sclerotiorum* (Purdy, 1979). These are often called ‘white mold,’ yet may be more accurately referred to as ‘Sclerotinia disease.’ In canola and soybean, the

disease caused by *S. sclerotiorum* is commonly referred to as 'stem rot', and represents one of the most important diseases in these crops.

1.3.3. Disease cycle and epidemiology

Three factors contribute to the success of *S. sclerotiorum* as a plant pathogen. Firstly, as noted above, the fungus can infect and reproduce on a large range of susceptible hosts. Secondly, high moisture can help to increase the chances of infection and production of inoculum during the growing season (Abawi and Grogan, 1979). Thirdly, the occurrence of drier soil conditions can favor survival of the pathogen, which can overwinter as sclerotia in the soil, or as mycelium and/or sclerotia on crop debris and living plants (Agrios, 1997).

The sclerotia of *S. sclerotiorum* germinate under favorable conditions in late spring or early summer. Germination can be myceliogenic, with the production of vegetative mycelium, or carpogenic, with the production of sexual fruiting bodies called apothecia. The type of germination depends largely on the prevalent environmental conditions. If nutrients are available and abundant, sclerotia may germinate and produce mycelium that infects the root tissues, causing crown rot and root rot of carrots (Finlayson et al., 1989). Furthermore, myceliogenic germination is more common in sclerotia that are melanin-deficient ('tan sclerotia') (Huang, 1981; 1983) or immature (and are therefore not completely melanized) (Huang and Kozub, 1994). This type of germination can also be induced in sclerotia cultured on medium amended with the melanin inhibitors tricyclazole and pyroquilon (Huang et al., 1993), or if normal black sclerotia are

mechanically injured (Huang, 1985), heated, desiccated, frozen, or kept under high humidity (Huang and Kozub, 1991; Huang et al., 1998).

While carpogenic germination of sclerotia is most common, whether or not it occurs can depend on the geographic origin of particular isolates (Huang and Kozub, 1991) and on the temperature at which the sclerotia formed (Huang and Kozub, 1989). The amount of soil moisture can also be an important factor in determining whether carpogenic germination of sclerotia occurs (Morrall, 1977). Temperatures of 4°C (Smith and Boland, 1989) or 10°C (Huang and Kozub, 1989; 1991), representing a cold-conditioning period, are usually required to trigger carpogenic germination if sclerotia are formed at temperatures higher than 20°C. Upon carpogenic germination, a stipe emerges from the sclerotium, with an apothecium produced at its tip. The top surface of the apothecium consists of a hymenium or fertile layer, made up of asci containing eight ascospores each. The ascospores are physically or mechanically discharged from the surface of the hymenium in an upward direction; the simultaneous discharge of millions of ascospores can appear as a smoke-cloud to the naked eye.

Canola crops, ascospores escaping from the canopy can be carried by wind or air currents to fields as far as several kilometers away. This type of dispersal can result in the development of local epidemics of stem rot. The ascospores are covered with sticky mucilage, which facilitates adhesion to the substrate on which they land (Clarkson et al., 2003). Upon landing, the ascospores will germinate; those landing on flowers or senescing tissues will quickly colonize these tissues, which provide a readily available source of nutrients. When infected petals are

shed by the canola plants, they land on or adhere to the leaves or stems. In canola, those infected petals landing on the leaf axils, which often contain free water and are adjacent to the stems, most often initiate infection of the stem. Infection of the leaves can also occur (Bolton et al., 2006). Under moist conditions favorable for infection, the fungal mycelium will continue to spread in the stem and other healthy tissue, and will sometimes infect tissues of other plants that are in direct contact with infected tissues. Further spread of the disease can be curtailed by the onset of dry conditions.

New sclerotia, which in canola are generally formed inside the infected stems, will fall to the soil at harvest and serve as inoculum for the next season, thereby completing the disease cycle. Thus, stem rot is a monocyclic disease, since no asexual spores are produced by *S. sclerotiorum* that could serve as secondary inoculum (Rimmer et al., 2003). In addition to the survival of the pathogen in the form of sclerotia, the fungus can also overwinter as mycelium in infected plant tissues. In western Canada, however, this mycelium rapidly loses its viability in the spring and early summer (Huang and Kozub, 1993b).

Sclerotinia stem rot can also be a seedborne disease; seeds may be contaminated with fungal sclerotia, which are easy to remove through cleaning, or they may be infected internally by the fungal mycelium.

1.3.4. Disease control

There are no effective cultural methods to completely manage Sclerotinia diseases, although some cultural practices tend to reduce their impact, including the utilization of pathogen-free or fungicide-treated seeds (Tu, 1989), increasing

spacing between and within rows, decreasing the seeding rate to reduce secondary spread (Hoes and Huang, 1985; Tu and Zheng, 1997), deep burying of the sclerotia to prevent carpogenic germination (Williams and Stelfox, 1980), or high levels of irrigation after harvest to stimulate the decomposition of sclerotia in the soil (Teo et al., 1989). Crop rotation is another strategy for the control of Sclerotinia diseases, although the incidence of stem rot on canola did not appear to be significantly reduced in a 3- to 4-year rotation (Williams and Stelfox, 1980; Morrall and Dueck 1982). During harvest, recommended strategies to minimize the risk of infection include direct combining of the crop, swathing only mature stands, and avoiding swathing if rain is in the immediate forecast. Furthermore, avoiding heavy or compact swaths, and the use of a high cut to allow for better drying are also useful in reducing field losses caused by *S. sclerotiorum* (Canola Council of Canada).

Treatment with foliar fungicides remains the principal method for control of stem rot of canola on the Canadian prairies. Currently, the fungicides Proline 480 (prothioconazole), Ronilan EG (vinclozolin), and Rovral (iprodione) are amongst the registered treatments (Alberta Crop Protection Guide, 2009). These are usually applied as aerial sprays at the full bloom stage, in order to protect the petals from ascospore infection. In order to minimize unnecessary applications of fungicides, several stem rot forecasting systems have been developed. Many factors influence forecasts and their relationship to actual disease levels. Some factors that must be considered when forescasting for the risk of Sclerotinia

disease include the field history (Turkington et al., 1988), rainfall, soil moisture (Hunter et al., 1984), and canopy density (Thomas, 1984).

Different approaches have been taken to develop forecasts for Sclerotinia stem rot. A checklist for predicting disease epidemics in oilseed rape has been available for many years (Thomas, 1984), in which host, pathogen, and environmental risk factors for stem rot are assessed in a questionnaire (Canola Council of Canada). The responses to the questionnaire are then used to predict the relative risk of disease development. Another forecasting system was developed that uses the incidence of petal infection as a proxy for risk of disease (Turkington et al., 1991; Turkington and Morrall, 1993). Briefly, rates of canola petal infection are used to predict the amount of inoculum present and the risk of stem rot in a particular crop. A commercial petal testing kit was developed for growers based on this forecasting system, and is available for purchase (Discovery Seed Labs Inc., Saskatoon, SK). Bom and Boland (2000) created a model that incorporated both petal infestation and soil moisture, which could correctly predict disease development more often than the model using petal infestation alone (Turkington and Morrall, 1993). Nevertheless, the accuracy of both models was affected by the timing of soil moisture measurements in relation to petal infestion, and the selection of threshold values for discriminating categories of soil moisture and petal infestation. Disease risk maps may also be a useful tool for predicting the possibility of stem rot development in canola fields (Canola Council of Canada). However, since these maps are based only on

weather-related parameters, namely temperature, and relative humidity, caution should be employed when interpreting the relative risk of disease.

Biological control represents another strategy to manage Sclerotinia disease, with most approaches focusing on the use of mycoparasitic fungi, bacteria, and insects. The fungus *Coniothyrium minitans* W.A. Campb. was the most effective agent in reducing the incidence of Sclerotinia wilt of sunflower (Huang, 1980). Although most biocontrol agents can restrict ascospore germination on water agar, inhibit mycelial growth on potato dextrose agar, and reduce host infection in growth chamber experiments (Mercier and Reeleder, 1987), they do not provide consistent control of white mold of bean in field tests (Inglis and Boland, 1992). There are a few examples of the use of antagonistic bacteria for the control of *S. sclerotiorum* in Canada. For instance, the bacterium *Bacillus cereus* Fr. & Fr. (strain alf-87A) reduced the incidence of basal pod rot of pea caused by ascospore infection (Huang et al., 1993). Other attempts to use bacteria for the control of *S. sclerotiorum* have also been initiated (de Freitas et al., 1999).

Hypovirulence has been observed in both *S. sclerotiorum* (Boland, 1992) and *S. minor* (Melzer and Boland, 1996). However, the use of hypovirulent isolates of *S. sclerotiorum* for the control of virulent strains of the fungus may be impractical (Boland, 1992), because of the high number of mycelial compatibility groups in this species (Kohn, 1995) and the importance of carpogenic germination in the epidemiology of above-ground diseases caused by the pathogen. The larvae of a fungus gnat (*Bradysia coprophila* Litner) were found to feed on mycelia and sclerotia of *S. sclerotiorum* (Anas and Reeleder, 1988). However, the feasibility

of using mycophagous insects to regulate or control pathogen populations and promote interaction with beneficial organisms in the field needs to be assessed further (Bardin and Huang, 2001). The use of organic and inorganic soil amendments represents another interesting approach for the biological control of Sclerotinia diseases (Bardin and Huang, 2001; Boland, 2004).

In economic terms, the deployment of genetically resistant host genotypes represents one of the most desirable strategies for plant disease control. In *Brassica* species, a significant amount of variation has been observed with respect to susceptibility to stem rot caused by *S. sclerotiorum* (Sedun et al., 1989). Although historically there have been few commercially available canola cultivars with genetic resistance to this pathogen, a canola hybrid ('45S51') with moderate levels of resistance to *S. sclerotiorum* was recently registered (Pioneer TechUpdate, 2009). This cultivar may serve as an important tool in managing stem rot on the prairies. Moreover, given the importance of petal infection in the stem rot disease cycle, the development of apetalous canola could be a genetically clever means of escaping infection (Rao et al., 1991; Jiang and Becker, 2003). Another strategy for the development of genetically resistant canola genotypes would involve the engineering of resistance-related genes into the host plants, thereby reducing losses through a transgenic approach (Stuiver and Custers, 2001). Ultimately, the sustainable management of stem rot of canola and other crops will require an integrated approach that incorporates cultural, chemical and biological methods, as well as disease forecasting and genetic resistance.

1.4. Host-pathogen interactions

1.4.1. Infection process

Infection by *S. sclerotiorum* usually occurs after the ascospores land on the petals or the infected tissue falls on the stems or leaves. The ascospore germinates and produces a germ tube, with an appressorium formed at the point of contact between the hypha and the plant tissue surface. This appressorium may be simple or complex in structure depending on the substrate (Tariq and Jeffries, 1984). The pathogen can usually penetrate directly through the host plant surface, with a penetration peg developing from the appressorium. The direct penetration of the cuticle was considered as a purely mechanical process in early studies (Boyle, 1921; Abawi et al., 1979). However, more recent ultrastructural studies suggested that enzymatic digestion of the cuticle also plays some role in the penetration process (Tariq and Jeffries, 1986). Just prior to penetration, a large vesicle develops at the tip of the appressorium, with a subcuticular vesicle forming in the host after penetration. Large hyphae fan out from this subcuticular vesicle, releasing cell wall degrading enzymes (CWDE) that dissolve the subcuticular wall of the epidermis (Hegedus and Rimmer, 2005). At the same time, *S. sclerotiorum* also secretes oxalic acid and other toxic substances, which further facilitate infection.

1.4.2. Oxalic acid

It is well known that some necrotrophic pathogens can secrete oxalic acid, a non-host specific toxin, to suppress the defense responses of the host plant (Dutton and Evans, 1996). Furthermore, oxalic acid plays complex and diverse

roles during the infection process, although it is a simple organic substance with limited chemical interactions (Hegedus and Rimmer, 2005). *S. sclerotiorum* has been found to secrete oxalic acid, which serves as an essential determinant of its pathogenicity (Maxwell and Lumsden, 1970; Noyes and Hancock, 1981; Marciano et al., 1983; Godoy et al., 1990; Zhou and Boland, 1999) and a virulence factor for the pathogen (Guimarães and Stotz, 2004). Evidence for the secretion of oxalic acid by *S. sclerotiorum* comes from studies with culture filtrates, manual injection of this compound into host tissues, the nature of symptom development, and its occurrence in infected tissues (Bateman and Beer, 1965; Maxwell and Lumsden, 1970; Noyes and Hancock, 1981; Marciano et al., 1983; Godoy et al., 1990).

There are currently several mechanisms proposed to explain the role of oxalic acid in the virulence of *S. sclerotiorum* (Dutton and Evans, 1996). Firstly, cell wall calcium (Ca^{2+}) can be chelated by oxalic acid, which can both compromise the function of Ca^{2+}-dependent defense responses and weaken the plant cell walls (Bateman and Beer, 1965). Secondly, the secretion of oxalic acid has been suggested to facilitate invasion because this compound may be directly toxic to hosts, presumably as a result of its acidity (Noyes and Hancock, 1981). Finally, the fungal enzymes (e.g., polygalacturonases) secreted by *S. sclerotiorum* in the infection court have maximal activities at lower pH values. It has therefore been postulated that oxalic acid might increase fungal virulence by shifting the pH in the infection court to a value better suited for enzymatic degradation of the plant cell walls (Bateman and Beer, 1965).

A number of studies support the above hypotheses. The calcium in the middle lamellae of the plant cell walls can be chelated by oxalic acid, allowing fungal polygalacturonases to hydrolyze pectates more readily (Bateman and Beer, 1965). Several fungal pectinolytic enzymes, such as the various endo- and exopolygalacturonases and pectin methylesterase (Lumsden, 1976; Riou et al., 1992a, b; Waksman et al., 1991), are most active in the acidic, low calcium environment resulting from the secretion of oxalic acid. The pH of infected tissue and culture media will decrease to pH 4 or 5 with increased oxalic acid concentrations. Since the optimal pH of extracellular enzymes produced by these oxalate-secreting fungi is generally below pH 5, the lowered pH of infected tissues enhances enzymatic activity (Bateman and Beer, 1965; Magro et al., 1984; Maxwell and Lumsden, 1970).

In addition, oxalic acid was also reported to directly enhance the activity of the endopolygalacturonase SSPG at low pH (Favaron et al., 2004). When the ambient pH falls to less than 3.8, expression of the polygalacturonase gene (specifically *Sspg1*) increases sharply (Rollins and Dickman, 2001; Cotton et al., 2003). The lower pH values may also interfere with the molecular interaction between plant inhibitor proteins (polygalacturonases) and cognate pathogen-derived polygalacturonases, enabling the latter to escape inactivation (Favaron et al., 2004). It is well established that an interaction between oxalic acid and polygalacturonases is important during colonization and symptom development in *Sclerotinia* diseases (Marciano et al., 1983; Maxwell and Lumsden, 1970). An

acidic environment may also serve as a signal to enhance the production of this virulence factor and coordinate the production of additional factors.

In one study by Godoy et al. (1990), mutants of *S. sclerotiorum* deficient for the production of oxalic acid were generated by UV irradiation. When these mutants regained their oxalate biosynthetic capacity, they exhibited normal virulence. Thus, this study provided evidence that oxalic acid is a determinant of pathogenicity (Godoy et al., 1990). Oxalic acid secreted by *S. sclerotiorum* has also been shown to suppress the oxidative burst, which serves as a defense mechanism for the host through the formation of reactive oxygen species (ROS) (Cessna et al., 2000). Oxalic acid also interferes with stomatal closure, firstly by disrupting the ABA-dependent process leading to the closing of stomates, and secondly by stimulating the accumulation of potassium and the hydrolysis of starch, both of which contribute to stomatal opening (Guimarães and Stotz, 2004). Differences in sensitivity to oxalic acid may partly explain the resistance to *S. sclerotiorum* among bean lines (Chipps et al., 2005).

1.4.3. Events during the interaction between host and pathogen

Plants are composed of immobile cells embedded in rigid cell walls, and therefore specialized defensive cells cannot move in response to microbial infection, as occurs in animals (Ebel and Mithöfer, 1998). A defense system has evolved wherein each plant cell has the capacity to respond to attempted infection and to establish a defense response. Therefore, plants are immune to most potential pathogens in a growth environment. In nature, there are relatively few true host-pathogen pairs existing between susceptible plants and virulent

pathogens (Ebel and Mithöfer, 1998). The plant cell can recognize microorganisms based on the generation of elicitors by these potential pathogens (Dixon et al., 1994). One of the first events detected in host cells artificially treated with fungal elicitors is the rapid and transient generation of activated oxygen radicals.

There are several models proposed to explain the biochemical basis of the interaction between plants and potential pathogens. One of these models hypothesizes that a ligand-receptor-like interaction between pathogen-produced signal compounds (elicitors) and corresponding plant receptors triggers the initial defense response. These elicitors may be cultivar-specific, species-specific, or of a more general type (non-host), depending on the given pathosystem. Race-specific elicitors are molecules encoded by phytopathogen avirulence (*Avr*) genes (de Wit, 1997). Specific plant cultivars carry genes conferring resistance to specific pathogen races harboring the corresponding avirulence genes (Heath, 1997). This type of interaction is also termed "gene-for-gene", wherein for each gene that confers resistance in the host there is a corresponding gene in the pathogen that confers virulence (Flor, 1971). The direct or indirect interaction of the A*vr* and host polypeptides triggering resistance has been shown through the cloning of plant resistance (R)-genes from host–pathogen model systems (Dangl, 1995). It has been proposed that R-gene products encode receptors capable of binding *Avr* products as ligands (Jones et al., 1994). The R-genes are also hypothesized to play a role as signaling intermediates in a reaction cascade, since the amino acid sequences of their products have homology to many structural

protein–protein interaction domains (de Wit, 1997). Non-race-specific elicitors, such as fungal or plant cell wall fragments released during the infection process, can also induce a host defense response (Blumwald et al., 1998).

A number of defense responses are induced by plant pathogen challenge, including the collapse of the challenged plant cells in a hypersensitive response (HR), which can be defined as a type of blocking necrosis by non-host plants against plant pathogens (Chester, 1993). The HR is considered a biochemical rather than a structural defense mechanism, and consists of the generation of ROS, the activation of defense-related genes, and structural changes in the cell wall (Agrios, 1997). As a result of these responses, lesions resulting from infection can be clearly delimited from the surrounding healthy tissue. A phenomenon known as systemic acquired resistance (SAR) is also observed in certain plants following the expression of the HR; SAR transiently increases resistance against subsequent pathogen challenge, and is also induced by certain elicitors (Agrios, 1997; Ebel and Mithöfer, 1998).

1.4.3. Elicitors and their perception

A number of substances with diverse chemical structures, collectively termed elicitors (Keen et al., 1972), have been reported to activate the various plant defense response systems (Darvill and Albersheim, 1984; Yoshikawa and Sugimoto, 1993; Ebel and Cosio, 1994; Boller, 1995; Hahn, 1996). The components of the host cell surface and host or pathogen-secreted metabolites of an oligosaccharide, glycoprotein, or fatty acid nature may all serve as elicitors.

Oligosaccharides were one of the earliest elicitors to be characterized in detail (Darvill and Albersheim, 1984). Four major classes of elicitor-active oligosaccharides have been identified: oligoglucan, oligochitin, and oligochitosan, which are of fungal origin, and oligogalacturonide, which is of plant origin (Côté and Hahn, 1994). Glucans were first isolated from the oomycete *Phytophthora sojae* Kaufm. & Gerd. by various treatments of the hyphal walls, and as a soluble fraction was spontaneously released from germinating zoospores of *P. sojae* (Ayers et al., 1976; Waldmüller et al., 1992). A major polysaccharide constituent of the hyphal walls of most of higher fungi is chitin (Bartnicki-Garcia, 1968). Chitin elicits a number of plant defense responses, including lignification (Pearce and Ride, 1982), and phytoalexin synthesis (Ren and West, 1992). Oligogalacturonides can also act as endogenous elicitors; these may be released from homogalacturonan, a pectic polysaccharide and an important structural component of the primary cell walls of higher plants, during pathogen attack (Côté and Hahn, 1994).

Glycopeptides and proteins that display different degrees of host-specificity have been identified, including glycoproteins. Glycopeptides and free N-linked oligosaccharides derived from glycoproteins are also active as elicitors in several cases (Anderson, 1989; Ebel and Cosio, 1994; Boller, 1995). The carbohydrate moieties were found to be responsible for the elicitor activity of various glycoproteins from *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cav. (Coleman et al., 1992) and *Puccinia graminis* Pers. (Kogel et al., 1988), whereas the protein portion or even an oligopeptide moiety elicited defense

responses in the *P. sojae*/parsley interaction (Nürnberger et al., 1994; Sacks et al., 1995). Glycoprotein elicitors have been shown to remain strictly localized to the area of application, where the HR occurs and signaling is initiated between the elicitor-treated and surrounding cells (Dorey et al., 1997). Plants can also perceive peptides and proteins alone (without a carbohydrate moiety) as elicitors of defense responses. A HR as well as SAR against fungal and bacterial pathogens in certain plant genotypes is induced by these types of proteins (Ricci et al., 1989; Kamoun et al., 1993). Similarly, the small extracellular proteins secreted by *Phytophthora* species are perceived in this manner by the host (Ricci et al., 1992; Kamoun et al., 1994; Boissy et al., 1996).

1.4.5. Signal transduction

As a consequence of the perception of elicitors, a number of rapid reactions are detectable in plant cells, including enhanced ion fluxes across the plasma membrane, formation of reactive oxygen intermediates, changes in protein phosphorylation, and lipid oxidation (Ebel and Mithöfer, 1998). Various physiological stimuli (Ward et al., 1995), including elicitors (Mathieu et al., 1991; Kuchitsu et al., 1993), have been studied with respect to plasma membrane depolarization in plant cells. Numerous mechanisms such as nonselective channels activation or modulation of anions (Ca^{2+}, K^{+}) or inhibition of proton pumps, can result in plasma membrane depolarization (Ward et al., 1995). Furthermore, the elements of elicitor-induced signal transduction may be represented by protein kinases and phosphatases, which convert receptor signals into cell-specific responses (Boller, 1995).

The oxidative burst associated with the generation of ROS is a rapid response of plants to the perception of elicitors and/or pathogen infection, and is a characteristic feature of the HR (Lamb and Dixon, 1997). Lipoxygenases or ROS can mediate the rapid oxidation of lipids in the cells of many plant species following elicitor-treatment (Rogers et al., 1988; Slusarenko et al., 1991). The expression of various genes in several plant systems can be triggered by infection, mechanical wounding, or treatment with numerous elicitors (Creelman and Mullet, 1997; Doares et al., 1995; Gundlach et al., 1992; Mueller et al., 1993; Nojiri et al., 1996; Reinbothe et al., 1994). Given their limited mobility, oligosaccharide and protein elicitors are not likely to be directly responsible for long-distance signaling (Aldington and Fry, 1993; Boller, 1995). Systemic intercellular signals, however, could be generated in plants by some immobile elicitors (Schaller and Ryan, 1995; Ryals et al., 1996).

1.4.6. Gene expression in the *B. napus*/*S. sclerotiorum* interaction

A number of studies have been conducted to understand the interactions between *S. sclerotiorum* and some of its main hosts. For instance, two cDNA libraries were constructed from fungal mycelium or *B. napus* stem tissue colonized by *S. sclerotiorum*, and over two thousand expressed sequence tags (ESTs) were generated (Li et al., 2004). Sequence analysis of the cDNAs obtained revealed that many of these encoded pathogenicity and signal transduction factors related to pathogenesis, including endo- and exo-polygalacturonases, as well as several metabolite transporters. In another study, *B. napus* genes expressed in response to challenge with *S. sclerotiorum* were

screened to identify possible quantitative trait locus (QTL) associated with resistance to this pathogen (Liu et al., 2005). Genes expressed differentially in *B. napus* in response to challenge with *S. sclerotiorum* have also been identified by cDNA microarray analysis (Zhao et al., 2007; Yang et al., 2007).

1.5. Proteomics in plant pathology

Proteomic analysis is a powerful approach that can provide a systematic understanding of molecular events associated with particular biological processes. Many proteome-level studies have begun to appear in the literature in recent years, especially in the area of plant pathology. Proteomic studies may provide novel insights and contribute to the understanding of pathogen biology and the host response to pathogen challenge. Ultimately, such increased knowledge may facilitate the development of improved strategies for integrated plant disease management.

1.5.1. Proteomic investigations of plant pathogens

In one proteomic study of a bacterial plant pathogen, a comprehensive profile of proteins secreted by *Erwinia chrysanthemi* (Burkholder et al.) was obtained (Kazemi-Pour et al., 2004). Of the approximately 100 protein spots found on the resulting proteome-reference map, 25 spots were identified as unique under uninduced culture conditions (e.g. Luria-Bertani media) among a total of 55 spots identified under different culture conditions. Inactivation of the enzyme luxS, which is responsible for the germination of a molecule (Autoinducer-2) used in quorum sensing by many bacterial species, was shown to have a strain-dependent

impact on the intracellular proteome, secreted proteins, motility and virulence of *E. carotovora* (Jones) Bergey et al. on potatoes (Coulthurst, et al., 2006). A comprehensive analysis of the secretome of *Xanthomonas campestris* (Pam.) Dow. revealed that many of the identified proteins were degradative enzymes likely involved in the infection of susceptible hosts (Watt et al., 2005). A comparative genetic and proteomics analysis resulted in the identification of a biomarker to distinguish virulent and avirulent strains of *Pantoea stewartii* (Smith) Mergaert et al. (Wu et al., 2007).

An investigation of the proteome of the fungal pathogen *Magnaporthe grisea* (T.T. Hebert) M.E. Barr, causal agent of rice blast, resulted in the identification of a number of proteins associated with appressorium formation (Kim et al., 2004b). Proteins associated with regulation of appressorium development were also identified in *P. infestans* (Mont.) de Bary, the causal agent of late blight of potato (Grenville-Briggs et al., 2005). An inventory of cell wall-associated proteins in *P. ramorum* Werres, de Cock & Man in't Veld, the cause of sudden oak death, revealed a total of 17 proteins important for pathogenicity (Meijer et al., 2006). An annoted proteome map of conidiospores from the barley powdery mildew pathogen *Blumeria graminis* (DC.) Speer has also been published (Noir et al., 2009). Proteome maps have also been developed for *Ustilago maydis* (DC.) Corda, causal agent of corn smut (Bohmer et al., 2007), *Botrytis cinerea* (de Bary) Whetzel, a necrotrophic and ubiquitous plant pathogen with a wide range host range (Fernández-Acero et al., 2006; 2007), and *Penicillium expansum* Link, a mycotoxigenic fungus associated with fruit decay (Qin et al., 2007). The

secretome of *F. graminearum* [*Gibberella zeae* (Schwein.) Petch], a devastating pathogen of wheat, maize and other cereals, was also characterized and shown to consist of a higher concentration and more diverse collection of proteins when the fungus was grown on plant cell wall extracts versus glucose (Phalip et al., 2005). More recently, the secretomes of virulent and avirulent isolates of the fungus causing tan spot of wheat, *Pyrenophora tritici-repentis* (Died.) Drechsler, were compared, revealing major quantitative and qualitative differences in the proteins produced (Cao et al., 2009). Perhaps of greatest interest, however, is the analysis of the mycelial proteome and secretome of *S. sclerotiorum* (Yajima and Kav, 2006), the fungal pathogen on which this thesis is focused. The information provided by that analysis will serve as an important resource as we examine the canola/*S. sclerotiorum* interaction on a molecular level.

1.5.2. Proteomic investigations of plant responses to fungal pathogens

Proteomic analyses have also contributed to the understanding of plant biology in recent years, including their responses to biotic and abiotic stresses (Cánovas et al., 2004; Rossignol et al., 2006). Proteomics, combined with transcriptomics, have been widely applied as tools for the identification of proteins that may play roles in plant defense responses to various pathogens (Mehta et al., 2008). The identification of such proteins is critical not only to the understanding of the defense response itself, but also to the interaction between plant and pathogen.

A proteomic investigation of wheat leaf rust, caused by the fungus *P. triticina* Erikss., revealed proteins with known and unknown functions as being

differentially abundant in the wheat host after pathogen challenge (Rampitsch et al., 2006). A proteomic experiment to study the wheat response to infection by *F. graminearum* was also carried out (Zhou et al., 2006). Another proteome-based investigation was conducted to examine the differential expression of proteins from rice leaves and suspension-cultured cells in response to inoculation with the rice blast fungus, *M. grisea* (Kim et al., 2003; 2004a). Similarly, comparison of the proteomes of different rice lines revealed a complex response to *M. grisea* challenge that involved the induction of proteins from various defense-related pathways (Lee et al., 2006). The leaf proteomes of two different pea genotypes resistant to *Erysiphe pisi* DC., cause of powdery mildew, were also compared in order to understand the mechanisms of this resistance (Curto et al., 2006). Many other studies have examined the response of hosts to infection in a variety of different pathosystems, including: root tissues of *Medicago truncatula* Gaertn. inoculated with *Aphanomyces euteiches* Drechsler (Colditz et al., 2004), maize embryos infected by *F. verticillioides* (Sacc.) Nirenberg (Campo et al., 2004), and tomato xylem sap after colonization by *F. oxysporum* Schltdl. (Houterman et al., 2007).

Proteomic analysis of the interaction between canola and some of its major pathogens is an area of particular interest. The response of two canola genotypes exhibiting differential reactions to infection by *A. brassicae*, the cause of Alternaria black spot, was investigated at the proteome level (Sharma et al., 2007). Similarly, leaf-proteome level changes in a susceptible *B. napus* genotype and a resistant *B. carinata* genotype were investigated, in order to understand the

impact of infection by the blackleg pathogen, *L. maculans* (Subramanian et al., 2005). Proteome-level changes in the proteomes of *B. napus* and *B. carinata* were further compared during infection by *L. maculans* (Sharma et al., 2008). A proteomic analysis of canola roots infected with *P. brassicae*, the causal agent of clubroot of crucifers, was also conducted (Cao et al., 2008). These studies have revealed novel information about the specific pathosystems involved. As such, in the present work, the response of *B. napus* canola upon *S. sclerotiorum* infection will be examined in detail.

1.6. Objectives

The primary objective of the work described in this thesis was to improve the understanding of the molecular events associated with pathogenesis and virulence in the *B. napus*/*S. sclerotiorum* interaction. To achieve this objective, we used a largely proteomic approach, although a number of other approaches were also employed as necessary, including histology, quantitative (q)-PCR, and fungal transformation. Thus, in the first study, a proteomic analysis of the *B. napus*/*S. sclerotiorum* interaction was conducted, with fungal ingress examined by histological observation and the expression of selected genes validated by q-PCR. In the second study, the focus shifted from the effects of the pathogen on the host proteome to those mediated by oxalic acid, an important pathogenicity factor produced by *S. sclerotiorum*, in order to better understand the note of oxalic acid during *S. sclerotiorum* infection. In the third chapter, the potential role of a hypothetically secreted protein from *S. sclerotiorum* was characterized in gene

disruption experuments. The fourth and fifth studies were primarily focused on the development of sclerotia and associated exudates to identify the proteins associated with sclerotial development, because these resting structures play an important role in the disease cycle and survival of the pathogen. Collectively, the work conducted in this thesis serves to provide novel insights on stem rot of *B. napus*, which will contribute to our ability to manage this important disease in a sustainable manner.

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2. Proteomic changes in leaves of *Brassica napus* L. as a result of *Sclerotinia sclerotiorum* challenge*

2.1. Introduction

Canola (*Brassica napus* L.) is an economically important oilseed crop that is cultivated worldwide, including in Canada and the USA. Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is one of the most serious diseases affecting canola and has the potential to significantly limit yields (del Río et al., 2007). In addition to being able to infect canola, *S. sclerotiorum* is a necrotrophic, generalist parasite capable of infecting approximately 400 plant species (Boland and Hall, 2007). In canola, outbreaks of Sclerotinia stem rot generally occur near the end of flowering, late in the growing season. Infection is initiated by airborne ascospores, which land on and infect the flower petals under favorable conditions (i.e., high moisture and high temperature); infected petals fall on leaves and leaf axils, from which the pathogen can grow into the host stem, forming lesions that may completely girdle the stem and cause plant death (Rimmer et al., 2003). Black sclerotia are eventually formed within the infected canola stems, which serve as survival structures for *S. sclerotiorum* and as a source of inoculum in subsequent seasons.

The biochemical and molecular events occurring in host plant tissues during disease progression are not clear. In the past, research on the molecular aspects of pathogenicity in *S. sclerotiorum* mainly focused on the roles of oxalic acid and cell-wall-degrading enzymes (CWDEs) such as glycosidases, pectinases,

*A version of this chapter has been published. Liang, Y., Srivastava, S., Rahman, M.H., Strelkov, S.E., Kav, N.N.V. 2008. J. Agric. Food Chem. 56, 1963-1976.

xylanases (Annis and Goodwin, 1997), and polygalacturonases (PGs) (Cotton et al., 2003). The secretion of oxalic acid by *S. sclerotiorum* has been found to be an essential determinant of its pathogenicity in bean and sunflower (Godoy et al., 1990), and the compound is considered to be a crucial pathogenicity factor produced by the fungus (Guimarães and Stotz, 2004). In addition to oxalic acid, the pathogen also secretes CWDEs that can facilitate penetration and degradation of host cell walls (Riou et al., 1991). Among the CWDEs, PGs are pectinases that can degrade unesterified pectate polymers present in plant cell walls and for which important roles as virulence factors have been demonstrated in a few pathosystems (Bolton et al., 2006). In addition, a recent analysis of the proteins secreted by this pathogen (the secretome) has revealed additional proteins that may be important during pathogenesis (Yajima and Kav, 2006).

Although there are a number of reports in the literature that describe the importance of oxalic acid and CWDEs for infection and pathogenesis by *S. sclerotiorum*, there are very few reports describing detailed molecular changes that accompany infection in the host. In fact, only two papers are available in the literature that have employed genomics-based techniques to identify plant and fungal genes that are modulated during *B. napus*–*S. sclerotiorum* interactions (Li et al., 2004; Liu et al., 2005). The first was an expressed sequence tag (EST) analysis of fungal genes expressed during growth in pectin medium or in infected plant tissue, which revealed a number of fungal genes important for pathogenesis (Li et al., 2004). The second study involved an investigation into changes in gene expression in the host (canola) tissue accompanying *S. sclerotiorum* infection and

which identified a number of plant genes that were modulated during infection (Liu et al., 2005). Although genomics-based investigation of host–pathogen interactions can provide valuable information into the changes in gene expression, an investigation into changes in protein abundance is also important in order to identify those proteins that are essential during such interactions. This is because there is often a poor correlation between transcript and protein abundance (Gygi et al., 1999). In this post-genomics era, proteomics-based approaches employing two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) are widely used to investigate various plant processes, including biochemical and physiological responses to abiotic or biotic stresses at the proteome level (Kav et al., 2004; Subramanian et al., 2005; Sharma et al., 2007; Jain et al., 2006; Zhou et al., 2006).

The identification and characterization of host proteins having levels affected by pathogen challenge are important in elucidating their roles in mediating the host response and is one of our long-term objectives. In this paper, we describe the changes in the leaf proteome of *B. napus* after infection by *S. sclerotiorum*, which was investigated using 2-DE and tandem MS. We have identified 32 proteins having levels that were modulated significantly at various time points after pathogen challenge, and the roles of these proteins are discussed within the context of the stem rot pathosystem.

2.2. Materials and methods

2.2.1. Fungal culture growth and inoculation of canola

Brassica napus and *Arabidopsis thaliana* seeds were sown in plastic trays with 32 cell packs containing Metro Mix 290 (Grace Horticultural products, Ajax, ON, Canada). Plants were grown in the greenhouse (16 h photoperiod, 22 ^{o}C day/18 ^{o}C night) for 18 days. *A. thaliana* seeds were vernalized by placing the trays in a dark room at 4 ^{o}C for 2 days. All plants were placed in a humidity chamber for 24 h prior to inoculation. Cultures of *Sclerotinia sclerotiorum* were grown on potato dextrose agar (PDA; Becton Dickinson, MD) and incubated at room temperature for 3 days. Actively growing mycelia from the edge of the colony were subcultured onto fresh PDA media and grown for an additional 2 days, after which mycelial plugs (5 mm) were excised from the cultures and used to inoculate leaves of *B. napus* (two true leaves) and *A. thaliana* (all the true leaves), which were gently wounded by scratching with a pipette tip. Similarly, control leaves were wounded and inoculated with PDA plugs alone. After inoculation, plants were returned to the humidity chamber for an additional 24 h, after which they were transferred to and maintained in the greenhouse. Shoot samples from control and inoculated plants were harvested at 6, 12, 24, 36 and 48 h after inoculation, flash frozen in liquid nitrogen and stored at –80 ^{o}C. Three independent biological replicates were performed for each treatment; each biological replicate consisted of 10 plants at each time point.

2.2.2. Histology

The infected leaf material and controls were cut into small (5 × 10 mm) sections, containing a portion of the wounded region, and fixed at 4 ºC overnight in 50 mM phosphate buffer (pH 7.2) containing 4% paraformaldehyde by vacuum

infiltration. Subsequently, they were dehydrated in a graded ethanol series, changed to toluene and infiltrated with Paraplast (Fisher, Pittsburgh, PA). Paradermal sections (6 μm thickness) were cut, affixed to slides, deparaffinated with toluene (two changes of 5 min each), rehydrated to 50% ethanol, and stained with Aniline Blue in lacto-phenol (Larone, 1995) for 10 min. Stained sections were rinsed with water (three changes of 3 min each), counterstained with acidified Eosin Y (Dougherty, 1981) for 1 min, dehydrated and mounted with DPX (Electron Microscopy Sciences, Hatfield, PA) mounting medium. The sections were viewed with a Leica DM *RXA* microscope (Leica Microsystems, Wetzlar, Germany) and photographed using an Optronics digital camera with Macrofire software (Optronics, Goleta, CA).

2.2.3. Protein extraction

Pooled *B. napus* leaves (~300 mg) were ground to a fine powder in liquid nitrogen and then resuspended in 1.5 mL of actone containing 10% trichloroacetic acid (TCA; Fisher, Fair Lawn, NJ) and 0.07% dithiothreitol (DTT, Fisher). After incubation at –20 °C for 1 h, the suspension was vortexed and centrifuged (18 000*g*, 15 min, 4 ºC). After removal of the supernatant, the pellet was resuspended in 1 mL of ice-cold acetone containing 0.07% DTT and centrifuged as described above. This washing step was repeated an additional four times. The washed pellets were dried for 10 min in a Speedvac (Hetovac VR-1; Heto Lab Equipment A/S, Birkerod, Denmark) and resolubilized in 400 μL rehydration/sample buffer (BioRad, Mississauga, ON, Canada) containing 0.1% tributylphosphine (TBP, BioRad) and incubated overnight at 4 ºC. After incubation, the samples were

vortexed vigorously and centrifuged as described above, and the supernatants were transferred to fresh tubes and stored at –20 °C until analysis by electrophoresis. The protein samples were prepared twice from each of three independent biological replicates of leaf tissue for a total of 6 samples for each time point. Protein concentrations were determined using a modified Bradford assay (Bradford, 1967) with bovine serum albumin (BSA; Pierce Biotechnology, Rochford, IL) as the standard.

2.2.4. Two-dimensional gel electrophoresis

Isoelectric focusing (IEF) of extracted protein in the first dimension and separation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension were performed as previously described (Sharma et al., 2007). Briefly, 17 cm IPG (pH 4–7, Bio-Rad) strips were rehydrated overnight with 400 μg of protein in 300 μL rehydration/sample buffer. IEF was preformed using a PROTEAN IEF cell (Bio-Rad) to separate proteins based on difference in isoelectric points. The focused IPG strips were equilibrated in 5 mL equilibration buffer containing 6 M urea, 2% SDS, 0.37 M Tris-HCl, pH 8.8, 20% glycerol, and 130 mM DTT for 10 min and then incubated in the same buffer containing 135 mM iodoacetamide (IAA) for 10 min. The equilibration steps in both buffers were performed twice. For the separation of the focused proteins in the second dimension, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a PROTEAN II XI cell (Bio-Rad). The separated proteins were stained using a Colloidal Coomassie Blue Staining Kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions.

2.2.5. 2-D gel analysis and protein identification

Images of the 2-D gels were recorded using a GS-800 calibrated densitometer (Bio-Rad) and analyzed using PDQuest 2-D analysis software (version 7.3.1, Bio-Rad). A matchset was created from images of 12 gels (for each time point), which consisted of 2 extractions from each of the three biological replicates for the control and inoculated plants at each time point. Analysis of the 2-D gels was performed using the automated detection and matching tools of the PDQuest software, and artifacts were removed by manual refinement of the matched spots. Replicate groups for control and inoculated gels were created and analyzed using the Student's *t*-test feature of the software, in order to identify those spots that showed statistically significant ($p < 0.05$) differences in intensities. The quantities of these significantly altered spots were determined using the spot quantification tool, and fold-changes were calculated. Among the statistically significant ($p < 0.05$) spots, only those that showed reproducible changes (up- or down-regulation) in all of the replicates were selected for further analysis using tandem MS. Electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI-q-TOF MS/MS) experiments were performed at the Institute for Biomolecular Design (IBD), University of Alberta, using a Micromass Q-TOF-2 mass spectrometer (Micromass, Manchester, UK) as described previously (Yajima and Kav, 2006). Data-dependent MS/MS acquisition was performed for peptides with a charge state of two or three, and the data were processed using the Mascot (Matrix science Inc., Boston, MA) search engine and the NCBI nonredundant protein database. Parameters that were utilized for the Mascot

search included carbamidomethylation of cysteine, possible oxidation of methionine, and one missed cleavage per peptide.

2.2.6. Quantitative real-time PCR

Primers and probes were designed using Primer Premier 3 software (Applied Biosystems Inc., Foster City, CA) to generate amplicons approximately 60 bp in size are listed in Table 2-1. Total RNA was extracted from control and inoculated pooled leaf samples for all the time points using RNeasy Plant Mini Kit (QIAGEN, Mississauga, ON, Canada). During RNA extraction, an RNase-free DNase (Qiagen) treatment was included to ensure complete removal of contaminating genomic DNA. First strand cDNA was synthesized by reverse transcription of 50 ng total RNA using the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed using TaqMan technology and the ABI PRISM 7700 (Applied Biosystems) DNA Sequence Detection System as previously described (Srivastava et al., 2007). qRT-PCR reactions were performed using 2 μL of 5 × diluted cDNA as template, 22.5 pmol of primers, 5 pmol of probe and 1 × TaqMan PCR Master Mix (Roche, Branchburg, NJ) in a reaction volume totalling 20 μL. The relative expression of the various genes was calculated using the delta–delta method, employing the following formula: relative expression = $2^{-[\Delta Ct\ sample\ -\ \Delta Ct\ control]}$ (Livak and Schmittgen, 2001). For all qRT-PCR experiments, the actin gene was used as the endogenous control. The relative expression in inoculated samples was normalized against the expression level in control samples (which were considered to be as 1). qRT-PCR reactions for samples from each biological replicate were performed in duplicate,

Table 2-1. Sequences of the primers and probes used for qRT-PCR analysis.

gene	accession no.	primers and probe
uroporphyrinogen decarboxylase	AY035057	F-5’-TGGGCAACCGAGCTTAGC-3’
(*A. thaliana*)		R-5 GAGAGGTAGGTTTGGGTGAGTTTG-3’
		P-5’-TGGATTTTGAGGAGTTTAGCT-3’
ATPase	NM_100523	F-5’-TGTTGGTGGACTCGGTGGTA-3’
(*A. thaliana*)		R-5-CAAACCGGTGATCTGTTGCA-3’
		P-5’-AGCTGCCGAAGAAG-3’
peroxidase	NM_111480	F-5’-TCCACCACGCCATTGTCA-3’
(*A. thaliana*)		R-5’-TAGGCACATGCTGCTGTGAAC-3’
		P-5’-AGAAAGTCGTCATCTTTGGT-3’
triosephosphate isomerase	NM_127687	F-5’-CGCTCCCCCAGAGGTGTT-3’
(*A. thaliana*)		R-5’-TTCCTCCAACGAAAAACTTTCC-3’
		P-5’- TGCCATGGCTGGATC-3’
fructose-bisphosphate aldolase	NM_120057	F-5’-GCGGTTTGGTTCCGATTGT-3’
(*A. thaliana*)		R-5’-TCAATGTCGTGTTCTCCATCCA-3’
		P-5’- AGCCAGAGATCTTG-3’
glyceraldehyde 3-phosphate dehydrogenase	ATHCPGAPBA	F-5’-GGAAGCTGTTGGAAGTGGAGAT-3’
		R-5’- TCATCAGCCGGGTTTGTCTT-3’
(*A. thaliana*)		P-5’- 6FAM-CTTTGGAGGATTTCTG-3’
glyoxalase I,	NM_105396	F-5’- AAGTTATGCTCCGTGTTGGTGAT-3’
(*A. thaliana*)		R-5’- TCCATTCCAAAAGCCTTCTCA-3’
		P-5’- CGATAGGGCCATAAAG-3’
actin	AF111812	F-5’- TGGGTTTGCTGGTGACGAT-3’
(*B. napus*)		R-5’- TGCCTAGGACGACCAACAATACT-3’
		P-5’- CTCCCAGGGCTGTGTT-3’
actin	AY120779	F-5’- GCCATTCAGGCCGTTCTTT-3’
(*A. thaliana*)		R-5’- ATCGAGCACAATACCGGTTGT-3’
		P-5’- TCTATGCCAGTGGTCG-3’

and the experiment was repeated at least twice for all the time-points.

2.2.7. Peroxidase and superoxide dismutase assays

Crude leaf extracts were prepared for the assay of peroxidase (POX) and superoxide dismutase (SOD) activities. Fresh, pooled leaves (~ 200 mg) from control (mock-inoculated), inoculated, and untreated (without wounding and inoculation) plants were ground in 1 mL of sodium phosphate buffer (60 mM, pH 7.0) and transferred to 1.5 mL tubes. The homogenates were centrifuged (20 000*g*) for 15 min at 4 ºC and the supernatants removed and used for POX and SOD assays. POX was assayed as described by Benkeblia and Shiomi (2004) with minor modifications. The reaction mixture (10 μL of 45 mM guaiacol, 10 μL of 200 mM H_2O_2, and 180 μL of 65 mM sodium phosphate buffer, pH 6.5) was mixed with 10 μL of crude enzyme extract, and the absorbance was immediately measured at 430 nm using a spectrophotometer. After incubation at room temperature for 10 min, the absorbance was measured again. One unit of enzyme activity was defined as a change in absorbance of 0.01 min^{-1} (Benkeblia and Shiomi, 2004). The POX enzyme assay was performed using leaf samples from three independent biological replicates and the whole experiment was repeated at least two times.

SOD enzyme activity was assayed by measuring the photochemical reduction of nitro blue tetrazolium [NBT] (Beauchamp and Fridovich, 1971) in a reaction mixture containing 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 0–20 μL enzyme extract in a total volume of 200 μL. To avoid delays in performing enzyme assays, ELISA plates

were used to accommodate multiple samples. The reaction mixture (except riboflavin) was added to all of the wells, riboflavin was added last, and the plate was placed 3 cm below a light source (two 34 W fluorescent lamps) for 10 min; the reaction was terminated by switching off the light source. The plates were covered with a black cloth until the absorbance was recorded at 560 nm and compared with the maximum absorbance in wells without the enzyme. The amount of enzyme extract corresponding to 50% inhibition of maximum color formation in enzyme-free extracts was considered to be one enzyme unit (Giannopolitis and Ries, 1977; Babitha et al., 2002).

2.2.8. Statistical analysis

Statistical analyses of proteome-level changes were performed using the Student's *t*-test feature of PDQuest software as described earlier and significance ($p > 0.05$) was calculated as per manufacturer's instructions. Analysis of POX, SOD activity and qRT-PCR results was performed using analysis of variance (ANOVA) with the mixed model procedure of SAS version 9.1 (Statistical Analysis system; SAS Institute Inc., Cary, NC).

2.3. Results and discussion

2.3.1. Morphology and histology

The appearance of *B. napus* leaves at various time points (6, 12, 24, 36 and 48 h) after inoculation with *S. sclerotiorum* are shown in Figure 2-1. Symptoms of leaf necrosis began to appear 12 h after inoculation, which spread relatively rapidly by 24 h, indicating that rapid invasion of the host tissue occurs between

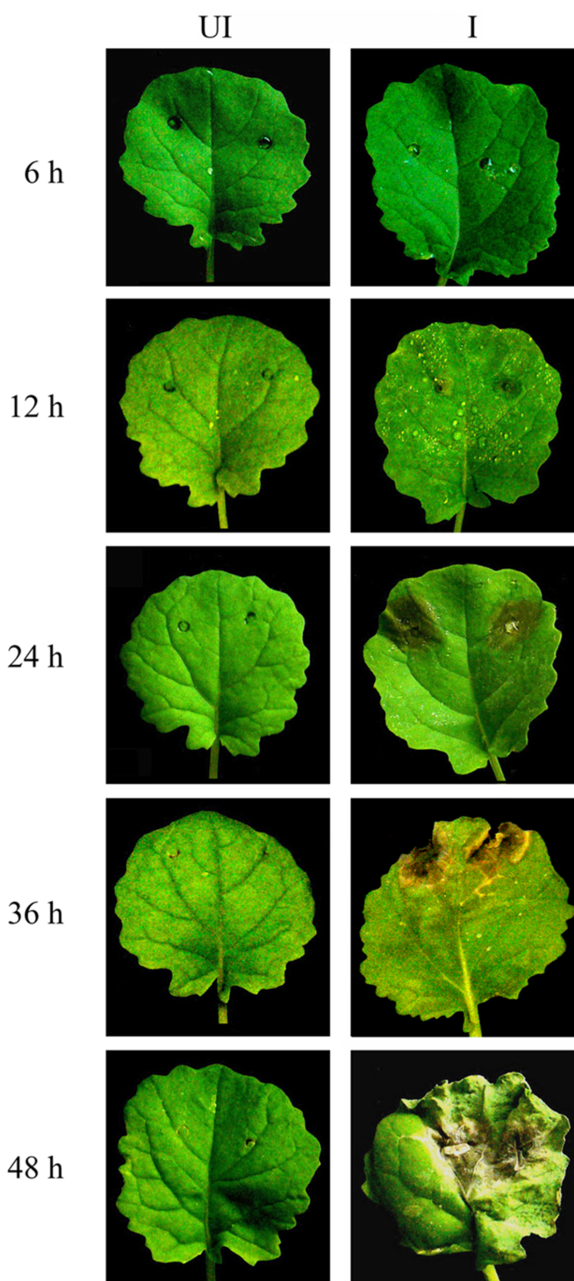


Figure 2-1. Appearance of *B. napus* leaves inoculated with *S. sclerotiorum*. Leaves are shown 6, 12, 24, 36 and 48 h after inoculation with the pathogen or mock-inoculation with an agar plug only. The labels UI and I refer to control and inoculated leaves, respectively.

12 and 24 h. To examine the invasion of leaf tissue by the fungus, we examined the uninoculated and inoculated leaves using light microscopy at the various time points indicated above. These results are illustrated in Figure 2-2. At 6 h after inoculation, there was no visible growth of the fungal mycelia in the host tissue. Although no mycelia were detectable, the cells in close proximity to the inoculation point demonstrated characteristics different from those of the cells that were distal to the infection site. These cells, especially the chloroplasts and the starch granules contained within them, and the adjacent cytoplasm stained more intensely with Aniline Blue and appeared darker compared to the cells more distal to the inoculation point. The control plant cells stained less intensely, and this phenomenon was also observed in all successive time points. The greater staining intensity of the cells may have been an indication of biochemical/physiological changes occurring before visual sympotom development. At 12 h after inoculation, fungal mycelia, stained pink with Eosin Y, could be seen in close proximity to the host tissue (Fig. 2-2), indicating the possible initiation of the infection process and the gradual establishment of the pathogen within the host. Once again, the mesophyll cells in close proximity to the fungal mycelia stained more intensely with Aniline Blue, whereas the cells in the noninfected region were lighter in color and had a purplish hue. Changes in histochemical properties, along with the accumulation of crystalline structures, preceding invasion of bean tissue by fungal mycelia have previously been reported (Lumsden and Dow, 1973). By 24 h after inoculation, the pathogen was

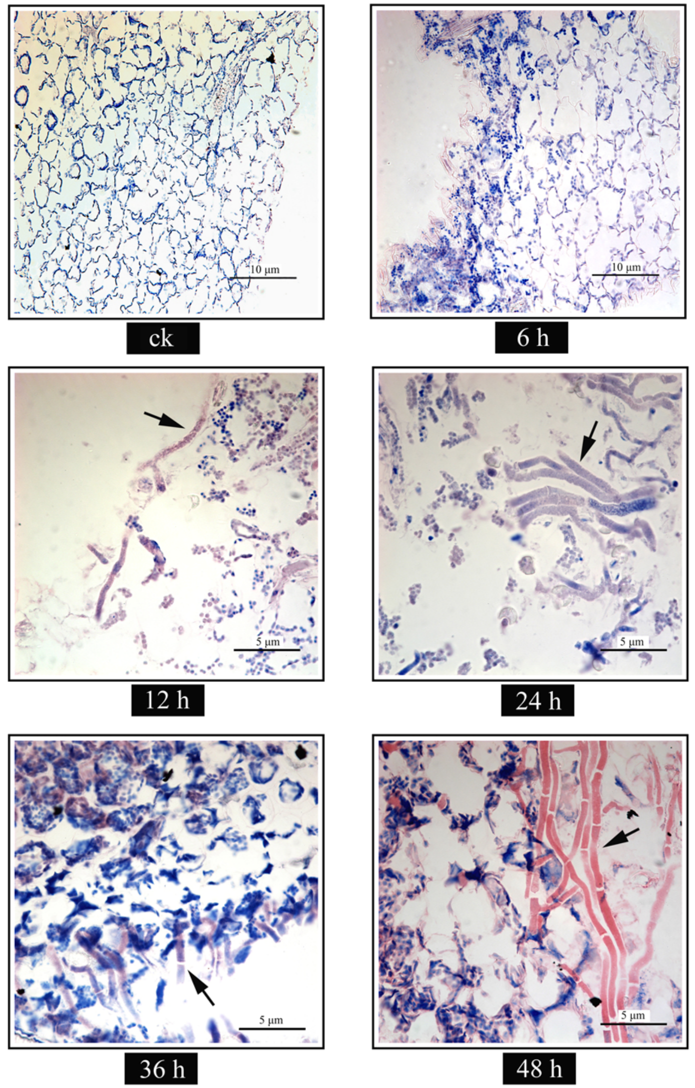


Figure 2-2. Light microscopy examining the invasion of *B. napus* leaves by *S. sclerotiorum* at various time points (6, 12, 24, 36 and 48 h) following inoculation or mock-inoculation (ck). Six micrometer thick paradermal sections of paraffin-embedded leaf tissue were stained with Aniline Blue in lactophenol and counterstained with Eosin Y. No fungal invasion (6 h), initiation of infection (12 h), and pathogen establishment and extensive mycelial growth (24–48 h) are apparent in these images. Arrows in the image point to fungal mycelia.

well established in the host tissue, and extensive mycelial growth was observed. The host tissue developed necrosis, and empty spaces in the tissue resulting from cell lysis could also be observed, which was also evident at 36 and 48 h after inoculation (Fig. 2-2). Our histological findings correlate well with the observed phenotypic changes after pathogen challenge at the various time points (6, 12, 24, 36 and 48 h). We observed no visible damage to the leaf tissue at 6 h, and the first symptoms appeared at 12 h (Fig. 2-1). The amount of fungal mycelia detectable in the host tissue increased at 24, 36, and 48 h after inoculation, as did the severity of macroscopic disease symptoms (Fig. 2-1 and 2-2).

2.3.2. Protein changes and identification of *S. sclerotiorum*-responsive proteins

Changes in the leaf proteome of *B. napus* resulting from challenge by *S. sclerotiorum* were characterized using 2-DE at 6, 12, 24, 36, and 48 h after inoculation, and representative images are shown in Figure 2-3. A total of 32 spots demonstrated significant ($p < 0.05$) differences (12 decreased and 20 increased) in intensities in samples from the inoculated leaf material, among which 4, 6, 8, 6, and 8 protein spots were identified at 6, 12, 24, 36 and 48 h after inoculation, respectively (Fig. 2-3 and Fig. 2-4). All of the proteins showing statistically significant ($p < 0.05$) changes in intensities after pathogen challenge were subsequently identified by MS/MS analysis and their identities are presented in Table 2-2. For most of the protein spots, a single protein was identified; however, for a few spots (9, 27, 28, and 29), multiple identities were obtained

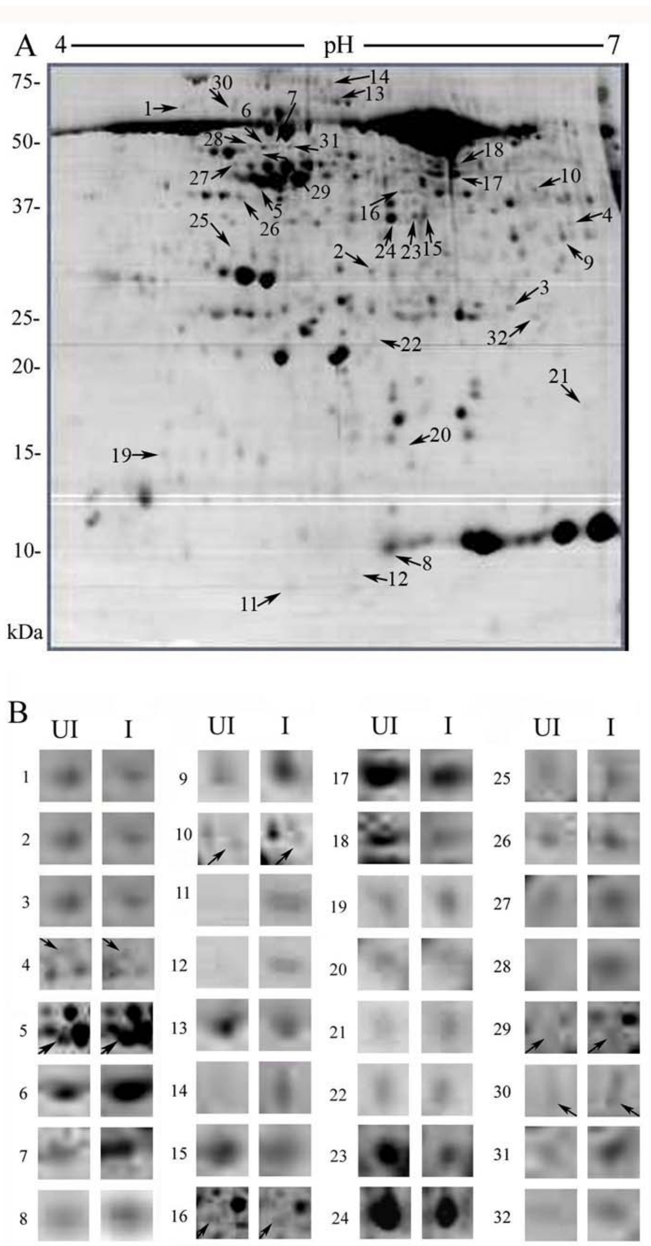
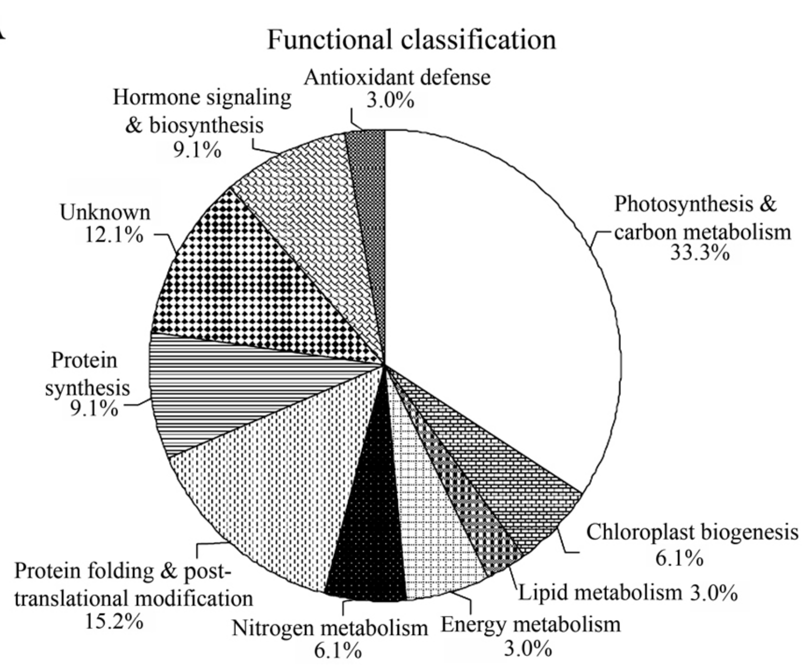


Figure 2-3. Proteome level analysis of *B. napus* leaves in response to inoculation with *S. sclerotiorum*: (A) representative image of *B. napus* leaf proteins separated by 2-DE and visualized with Coomassie Blue. [protein spots, selected from MS/MS analysis, having intensities that were significantly ($p < 0.05$) as a result of pathogen challenge are indicated by arrows and numbers]; (B) closer view of spots showing significant changes.



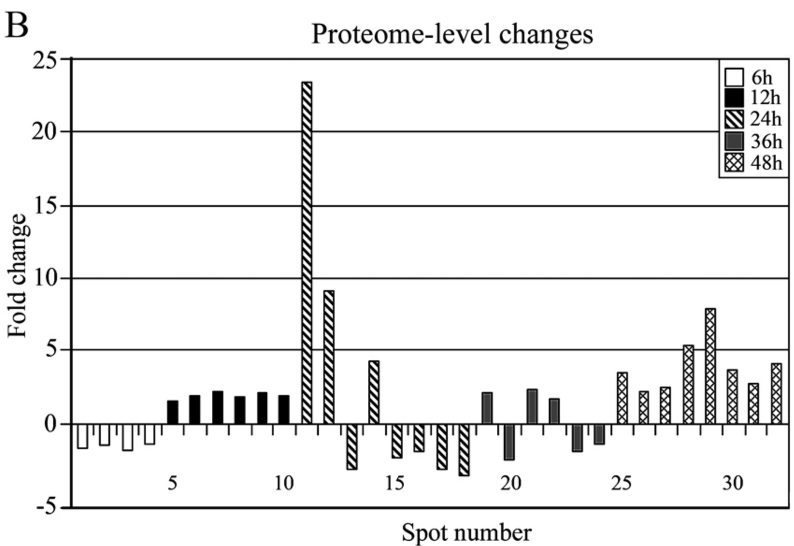


Figure 2-4. Functional classification (A) of proteins identified by tandem mass spectrometry and the fold changes in levels (B) following inoculation with *S. sclerotiorum*.

(Table 2-2). In those the molecular masses and/or isoelectric points were very close, making the unambiguous identification of these spots impossible. In addition, one spot (spot 7; Table 2-2), for which the score was below the threshold value, generated the same identity after repeated MS/MS analysis and was therefore included among the proteins identified. We have grouped the identified proteins based on their functional roles, which are discussed in detail in subsequent sections.

2.3.3. Functional classification of identified proteins

As indicated earlier, the proteins identified in this study as being significantly ($p < 0.05$) affected by *S. sclerotiorum* challenge were grouped according to their known intracellular functions (Fig. 2-4). It is evident that a large portion (33%) of the proteins have roles in photosynthesis and carbon metabolism. The second largest group of proteins (15%) was identified as those involved in protein folding and post-translational modifications, whereas 12% could not be classified based on the basis of available information. Other major categories were comprised of proteins involved in hormone biosynthesis and signaling (9%), protein synthesis (9%), energy metabolism (3%), chloroplast biogenesis (6%), nitrogen metabolism (6%), lipid metabolism (3%), and antioxidant defense (3%). The remainder of our discussion will focus on the role of proteins involved in (1) photosynthesis and metabolic pathways, (2) protein folding and modifications, (3) hormone signaling, and (4) antioxidant defense, becasue these proteins may have significant and important roles in mediating plant responses to the pathogen.

Table 2-2. Summary of the differentially expressed proteins identified from susceptible leaf tissue of *B. napus* after inoculation with *S. sclerotiorum*

spot	time point (h)	protein identity	MS/MS/PM[a] (%)	ESI-q-ToF score[b]	ESI-q-ToF peptide sequence (charge state)	accession no.[c]	M_r/pI	fold change
1	6	ATPDIL1-1; electron transporter/ isomerase/ protein disulfide isomerase [*Arabidopsis thaliana*]	3/3	106/50	K.IDASEETNR.E (2^+) K.IDASEETNR.E (2^+) K.AVQEYNGPR.E (2^+)	gi 15219086	55852/ 4.81	1.71 ± 0.26↓
2	6	unknown protein [*A. thaliana*]	4/10	114/49	R.TGQIVYK.K (2^+) K.MKPGFDPTK.G (2^+) K.MKPGFDPTK.G (2^+) K.DDELLQTDTK.T (2^+)	gi 18413869	27143/ 6.19	1.52 ± 0.12↓
3	6	triosephosphate isomerase 1 [*Zea mays*]	4/10	175/49	K.VIACVGETLEQR.E (2^+) R.EAGSTMDVVAAQTK.A (2^+) R.EAGSTMDVVAAQTK.A (2^+) R.EAGSTMDVVAAQTK.A (2^+)	gi 168647	27236/ 5.52	1.79 ± 0.25↓
4	6	malate dehydrogenase/ oxidoreductase [*A. thaliana*]	2/6	128/49	K.LFGVTTLDVVR.A (2^+) R.TQDGGTEVVEAK.A (2^+)	gi 15232468	36024/ 8.30	1.38 ± 0.15↓
5	12	ATP binding/kinase/ phosphoribulokinase/ uridine kinase [*A. thaliana*]	2/5	71/50	R.KLTCSYPGIK.F (2^+) K.FYGEVTQQMLK.H (2^+)	gi 15222551	44721/ 5.71	1.54 ± 0.20↑
6	12	JR1 [*A. thaliana*]	2/3	69/38	K.FDYEKDGK.I (2^+) K.IVSLEHGK.Q (2^+)	gi 30684083	48524/ 5.12	1.89 ± 0.34↑
7	12	JR1 [*A. thaliana*]	1/1	35/37	K.FDYEKDGK.I (2^+)	gi 30684083	40524/ 5.12	2.17 ± 0.30↑
8	12	ribulose bisphosphate carboxylase [*Brassica napus*]	4/16	124/50	R.EHGSTPGYYDGR.Y (3^+) R.EHGSTPGYYDGR.Y (2^+) K.TEYPNAFIR.I (2^+) R.IIGFDNNR.Q (2^+)	gi 17850	20499/ 8.23	1.81 ± 0.34↑
9	12	ferredoxin-NADP$^+$ reductase [*A. thaliana*]	3/12	124/50	R.EGQSVGVIADGIDK.N (2^+) K.DPNATVIMLATGTGIAPFR.S (3^+) K.DNTFVYMCGLK.G (2^+)	gi 8778996	39147/ 8.65	2.08 ± 0.38↑
		chain B, wild-type pea Fnr	2/7	75/50	R.LVYTNDAGEVVK.G (2^+) K.DNTFVYMCGLK.G (2^+)	gi 4930124	35060/ 6.54	
10	12	putative uroporphyrinogen decarboxylase [*A. thaliana*]	7/10	110/38	K.SYQTLCEK.Y (2^+) K.SYQTLCEK.Y (2^+) K.SYQTLCEK.Y (2^+) K.SYQTLCEK.Y (2^+) K.VLHALLQK.F (2^+) K.FTTSMITYIR.Y (2^+) R.DIAVQGNVDPGVLFGSK.E (2^+)	gi 14334768	43694/ 8.60	1.92 ± 0.34↑
11	24	unnamed protein product [*Sinapis alba*]	2/24	117/50	R.EHGSTPGYYDGR.Y (2^+) R.IIGFDNNR.Q (2^+)	gi 1345574	9768/ 6.10	23.43 ± 9.50↑
12	24	unnamed protein product [*S. alba*]	1/14	75/49	R.EHGSTPGYYDGR.Y (2^+)	gi 1345574	9768/ 6.10	9.11 ± 4.11 ↑
13	24	chaperonin precursor [*Pisum sativum*]	4/8	156/50	K.LADLVGVTLGPK.G (2^+) K.DTTTIVGDGSTQEAVNK.R (2^+) K.NAGVNGSVVSEK.V (2^+) K.YGYNAATGK.Y (2^+)	gi 806808	63287/ 5.85	3.13 ± 0.93↓
14	24	FTSH8; ATP-dependent peptidase/ ATPase/ metallopeptidase/ zinc ion binding [*A. thaliana*]	2/3	72/50	R.IVAGMEGTVMTDGK.S (2^+) K.ETMSGDEFR.A (2^+)	gi 42561751	73324/ 5.72	4.27 ± 1.56↑
15	24	fructose-bisphosphate aldolase [*A. thaliana*]	2/4	69/49	R.TAAYYQQGAR.F (2^+) R.ALQNTCLK.T (2^+)	gi 18399660	43075/ 6.18	2.37 ± 0.27↓
16	24	mRNA binding [*A. thaliana*]	5/11	156/50	R.FSEIVSGGGK.T (2^+) K.DCEEWFFDR.I (2^+) K.TVEIVHYDPK.A (2^+) K.DLLGWESK.T (2^+) K.TNLPEDLKER.F (2^+)	gi 15229384	44074/ 8.54	1.92 ± 0.22↓
17	24	glyceraldehyde 3-phosphate dehydrogenase B subunit [*A. thaliana*]	13/36	482/49	K.DSPLEVVVLNDSGGVK.N (2^+) K.YDSMLGTFK.A (2^+) K.IVDNETISVDGK.L (2^+) K.VLDEEFGIVK.G (2^+) K.GTMTTTHSYTGDQR.L (2^+) R.AAALNIVPTSTGAAK.A (2^+) R.AAALNIVPTSTGAAK.A (2^+) K.AVSLVLPQLK.G (2^+) R.VPTPNVSVVDLVINVEK.K (2^+) K.GLTAEDVNEAFR.K (2^+) K.VVAWYDNEWGYSQR.V (2^+) K.WPGAEAVGSGDPLEDFCK.T (2^+) K.WPGAEAVGSGDPLEDFCK.T (2^+)	gi 336390	43168/ 5.60	3.10 ± 0.73↓

Table 2-2. Continued

				ESI-q-ToF				
spot	time point (h)	protein identity	MS/MS/PM[a] (%)	score[b]	peptide sequence (charge state)	accession no.[c]	M_r/pI	fold change
18	24	methionine adenosyltransferase/ATP binding [*A. thaliana*]	10/20	262/50	R.EIGFISADVGLDADK.C (2^+) R.EIGFISADVGLDADK.C (2^+) K.NDGGAMIPIR.V (2^+) K.YLDDNTIFHLNPSGR.F (3^+) R.FVIGGPHGDAGLTGR.K (2^+) R.FVIGGPHGDAGLTGR.K (2^+) R.FVIGGPHGDAGLTGR.K (3^+) R.FVIGGPHGDAGLTGR.K (2^+) K.TGTIPDKDILVLIK.E (3^+) K.TAAYGHFGR.D (2^+)	gi 15228048	42927/ 5.76	3.65 ± 0.71 ↓
19	36	ribosomal protein L12 [*A. thaliana*]	4/16	125/49	K.IGSEISSLTLEEAR.I (2^+) K.IGSEISSLTLEEAR.I (2^+) R.ALTSLALK.E (2^+) K.ELIEGLPK.K (2^+)	gi 468773	19744/ 5.51	2.10 ± 0.27 ↑
20	36	eukaryotic translation initiation factor-5A [*B. napus*]	2/23	121/49	K.CHFVAIDIFTAK.K (2^+) K.SGFEEGKDVVVSVMSSMGEEQICAVK.E (3^+)	gi 40805177	17315/ 5.71	2.50 ± 0.59 ↓
21	36	peroxidase/ATPRXIIF/PRXIIF [*A. thaliana*]	3/15	101/49	K.FSTTPLSDIFK.G (2^+) K.DAIEFYGDFDGK.F (2^+) R.WSAYVEDGK.V (2^+)	gi 18397457	21546/ 8.99	2.33 ± 0.41 ↑
22	36	PBA1; endopeptidase/ peptidase/ threonine endopeptidase [*A. thaliana*]	4/9	168/49	K.ITQLTDNVYVCR.S (2^+) R.TVIINSEGVTR.N (2^+) R.TVIINSEGVTR.N (2^+) R.TVIINSEGVTR.N (2^+)	gi 15235889	25193/ 5.31	1.71 ± 0.28 ↑
23	36	AT4g38970/F19H22_70 [*A. thaliana*]	10/25	396/50	R.GILAMDESNATCGK.R (2^+) K.MVDVLVEQNIVPGIK.V (2^+) K.MVDVLVEQNIVPGIK.V (2^+) R.TAAYYQQGAR.F (2^+) R.TVVSIPNGPSALAVK.E (2^+) R.TVVSIPNGPSALAVK.E (2^+) R.YAAISQDSGLVPIVEPEILLDGEHDIDR.T (3^+) R.ALQNTCLK.T (2^+) K.YTGEGESEEAK.E (2^+) K.YTGEGESEEAK.E (2^+)	gi 16226653	43029/ 6.79	1.91 ± 0.32 ↓
24	36	fructose-bisphosphate aldolase [*A. thaliana*]	18/28	543/50	R.LDSIGLENTEANR.Q (2^+) R.LDSIGLENTEANR.Q (2^+) K.MVDVLVEQNIVPGIK.V (2^+) K.MVDVLVEQNIVPGIK.V (2^+) K.MVDVLVEQNIVPGIK.V (2^+) K.GLVPLVGSNNESWCQGLDGLSSR.T (2^+) K.GLVPLVGSNNESWCQGLDGLSSR.T (3^+) R.TAAYYQQGAR.F (2^+) R.YAAISQDSGLVPIVEPEILLDGEHDIDR.T (3^+) R.ATPEQVAAYTLK.L (2^+) R.ATPEQVAAYTLK.L (2^+) R.ATPEQVAAYTLK.L (2^+) K.YTGEGESEEAK.E (2^+) K.YTGEGESEEAK.E (2^+) K.YTGEGESEEAK.E (2^+) K.YTGEGESEEAK.E (2^+) K.YTGEGESEEAK.E (2^+) K.YTGEGESEEAK.E (2^+)	gi 18420348	43132/ 6.78	1.42 ± 0.13 ↓
25	48	putative glyoxalase [*Oryza sativa* (*japonica* cultivar group)]	2/6	88/41	-.MLHVVYR.V (2^+) K.FYTECLGMK.L (2^+)	gi 50933389	29720/ 4.99	3.50 ± 1.30 ↑
26	48	plastid-dividing ring protein [*Solanum tuberosum*]	3/9	198/38	K.VVGVGGGGNNAVNR.M (2^+) R.NVDTLIVIPNDR.L (2^+) K.DSGTAMLGVGVSSSK.D (2^+)	gi 47156057	44089/ 5.79	2.15 ± 0.76 ↑
27	48	RCA (rubisco activase) [*A. thaliana*]	3/9	186/38	K.MGINPIMMSAGELESGNAGEPAK.L (2^+) K.MCCLFINDLDAGAGR.M (2^+) R.VYDDEVRK.F (2^+)	gi 18405145	52347/ 5.87	2.47 ± 0.45 ↑
		plastidic glutamine synthetase precursor [*B. napus*]	8/13	175/38	K.WNYDGSSTGQAPGEDSEVILYPQAIFR.D (3^+) R.AAEIFSNK.K (2^+) R.AAEIFSNKK.V (2^+) R.EEGGFDVIK.K (2^+) R.HMEHISAYGEGNER.R (3^+) R.HMEHISAYGEGNER.R (3^+) R.HMEHISAYGEGNER.R (3^+) R.HMEHISAYGEGNER.R (3^+)	gi 1934754	47758/ 5.99	

Table 2-2. Continued

spot	time point (h)	protein identity	MS/MS/PM[a] (%)	ESI-q-ToF score[b]	peptide sequence (charge state)	accession no.[c]	M_r/pI	fold change
28	48	RCA (rubisco activase) [*A. thaliana*]	4/9	189/41	K.MGINPIMMSAGELESGNAGEPAK.L (3^+) K.MGINPIMMSAGELESGNAGEPAK.L (2^+) K.MCCLFINDLDAGAGR.M (2^+) R.VYDDEVR.K (2^+)	gi 18405145	52347/ 5.87	5.32 ± 1.92 ↑
		JR1 [*A. thaliana*]	3/7	68/41	K.FDYEKDGK.I (2^+) K.TSQPFGLTSGEEAELGGGK.I (2^+) K.IVSLEHGK.Q (2^+)	gi 30684083	48524/ 5.12	
29	48	unnamed protein product [*A. thaliana*]	1/3	117/40	R.GLAYDTSDDQQDITR.G (2^+)	gi 16471	52080/ 5.80	7.89 ± 2.71 ↑
		glutamate-ammonia ligase precursor [*B. napus*]	1/3	66/40	R.HMEHISAYGEGNER.R (3^+)	gi 296223	47714/ 6.16	
30	48	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*B. juncea*]	3/6	60/38	K.LNYYTPEYETK.D (2^+) R.DNGLLLHIHR.A (2^+) R.ESTLGFVDLLR.D (2^+)	gi 30959088	53436/ 5.88	3.74 ± 1.07 ↑
31	48	ADP-glucose pyrophosphorylase small subunit [*B. napus*]	3/6	42/38	R.AKPAVPLGANYR.L (2^+) K.IYVLTQFNSASLNR.H (2^+) R.SAPIYTQPR.Y (2^+)	gi 7688095	57294/ 5.87	2.76 ± 0.74 ↑
32	48	proteasome-like protein α subunit [*S. tuberosum*]	2/8	111/50	K.LLQTSTSSEK.M (2^+) K.TMDSTSLTSEK.L (2^+)	gi 77999303	27293/ 5.63	4.14 ± 1.46 ↑

[a] Number of peptides matched/ percent sequence coverage.

[b] Mascot score for the most significant hits/Mascot cut-off (threshold score)

[c] Accession numbers for proteins generated by the Mascot search

2.3.4. Metabolism

A number of the proteins found in our study are known to be involved in carbon metabolism, including spots identified (Fig. 2-3; Table 2-2) as ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco; spots 8 and 30), rubisco activase (RCA; spots 27 and 28), and phosphoribulokinase (PRK; spot 5). These proteins were up-regulated following inoculation. In contrast, protein spots identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH; spot 17), fructose bisphosphate aldolase (FBPase; spots 15 and 24), triosephosphate isomerase (TPI; spot 3), and malate dehydrogenase (MDH; spot 4) were down-regulated following pathogen-challenge (Fig. 2-3; Table 2-2).

Rubisco catalyzes the carboxylation and oxygenation of ribulose bisphosphate in the Calvin and photorespiratory cycles, respectively (Roy, 1989). Rubisco is one of the key and unique catalysts of the Calvin cycle, one of the central steps in the global carbon cycle (Hanson and Tabita, 2001). Rubisco activase (RCA), a nuclear-encoded chloroplast protein, which removes inhibitors from its catalytic sites, alters its conformation and activates rubisco *in vivo* (Andrews et al., 1995; Portis et al., 1995), and our proteome analysis also revealed an increase in intensities of two spots identified as RCA at 48 h after pathogen challenge. It is important to indicate at this point that spots 27 and 28, which were both identified as RCA, also generated an additional hit each (Table 2-2) and, therefore, it is possible that these spots may not be RCA. However, for spot 28, the Mascot score for the second hit is considerably lower than that of the first hit, which was for RCA. In addition, spot 11 (Fig. 2-3; Table 2-2), which

was identified as an unnamed protein, exhibited 98% homology with rubisco (data not shown). Thus our results indicate that rubisco, and perhaps RCA as well as PRK, which are involved in catalyzing reactions (PRK and rubisco) or activating enzymes (RCA) involved in the first stage of the Calvin cycle, may be increased as a result of *S. sclerotiorum* infection.

However, our studies also revealed a pathogen-induced decrease in the intensities of other enzymes involved in the Calvin cycle, including GAPDH, FBPase and TPI (Table 2-2). The subunit of the plastidic form of GAPDH, which was found to be down-regulated ~3-fold at 24 h after inoculation (spot 17; Fig. 2-3; Table 2-2), participates in the NADPH-dependent conversion of 1,3-bisphosphate glyceric acid to glyceraldehyde-3-phosphate (GAP). GAP and its isomer dihydroxyacetone phosphate (DHAP) are utilized in the biosynthesis of fructose 1,6-bisphosphate by FBPase, which was also observed to decrease in intensity at 24 h (spot 15; Fig. 2-3; Table 2-2), suggesting that the formation of hexose phosphate by the Calvin cycle may be reduced due to pathogen infection. In addition, spot 23 (Fig. 2-3; Table 2-2) also exhibited an approximate twofold decrease in intensity and generated peptides that matched FBPase. Incidentally, TPI, which catalyzes the isomerization of GAP to DHAP, was also observed to decrease in intensity, albeit to a lesser extent (1.8-fold) and at an earlier time point (6 h) (spot 3; Fig. 2-3; Table 2-2). Our results suggest that even though CO_2 fixation by rubisco may be increased in infected leaf tissue, subsequent reactions may be slowed down as a result of a decrease in the levels of GAPDH, TPI and FBPase. The exact significance of the *S. sclerotiorum*-induced increase in PRK

and rubisco, and the concomitant decrease in GAPDH, TPI, and FBPase remain unclear.

Some of the proteins identified are involved in energy metabolism and transport, including ADP-glucose pyrophosporylase (AGPase; spot 32; Fig. 2-3; Table 2-2). AGPase produces ADP-glucose, a substrate for starch synthesis in plants (Kleczkowski, 2000), and it has been suggested that this enzyme is tightly regulated by 3-phosphoglycerate and inorganic phosphate (Sanwal et al., 1968). The other protein that also increased following pathogen challenge was uroporphyrinogen decarboxylase (UROD; spot 10; Fig. 2-3; Table 2-2). UROD is a central enzyme in the tetrapyrrole biosynthetic pathway, which may have a role in mediating plant responses to pathogens (including tolerance). This suggestion is supported by the fact that the antisense of UROD in tobacco confers increased tolerance to tobacco mosaic virus (TMV) (Mock et al., 1999).

Nitrogen nutrition is critical to plants, and nitrogen deficiency often results in increased susceptibility to diseases (Pageau et al., 2006). Proteins involved in nitrogen metabolism, including glutamine synthetase (GS; spot 27) and glutamate ammonia ligase (spot 29; Fig. 2-3; Table 2-2), were identified in our studies, although as the number two hit in both instances. GS/glutamate ammonia ligase is a key enzyme that is responsible for the assimilation of ammonia, catalyzing the reaction of NH_3, ATP and L-glutamate to phosphate, ADP and L-glutamine (Zörb et al., 2004). Increased expression of GS during disease progression has been demonstrated in many host–pathogen interactions. These include pathosystems involving *Stylosanthes guianensis–Colletotrichum gloeosporioides* (Stephenson et

al., 1997), *B. carinata–Leptosphaeria maculans* (Subramanian et al., 2005), *Triticum aestivum–Puccinia triticina* (Rampitsch et al., 2006), and *T. aestivum–Fusarium graminearum* (Zhou et al., 2006). Furthermore, the inhibition of GS activity by a phytotoxin produced by *Pseudomonas syringe* (Langston-Unkefer et al., 1987) suggests a crucial role for this enzyme in mediating host–pathogen interactions. Because leaf necrosis affects nitrogen metabolism from assimilation to remobilization, which is normally accompanied by an elevation of GS (Ochs et al., 1999), it is possible that the increased level of GS observed in this study may positively affect the remobilization of nitrogen from those necrotic tissues.

2.3.5. Chaperones and post-translational modification of proteins

Molecular chaperones are a family of cellular proteins that mediate the correct folding of other proteins, but they themselves are not components of the final structures (Ellis and Hemmingsen, 1989). Under various stress conditions, chaperones play an important role in restoring the native conformation of unfolded proteins (Wilkinson and Gilbert, 2004). Our proteomics investigation revealed the identity of a few proteins with chaperone activity. These included protein disulfide isomerase (PDI; spot 1) and chaperonin precursor (spot 13; Fig. 2-3; Table 2-2). PDI acts as a molecular chaperone in maintaining proper protein folding and besides processing disulfide isomerase activity, catalyzes the refolding of many proteins (Schultz-Norton et al., 2006). Within the context of plant responses to pathogens, it has been reported that nitric oxide (NO), an essential component of certain defense-signaling cascades, is regulated by PDI (Zai et al., 1999). In addition, it has been reported that upon *Mycosphaerella*

graminicola infection to wheat, an observed induction of PDI that occurred within 3 h was greatest in resistant lines (Ray et al., 2003). Chaperonins are molecular chaperones, which are present in the plastids, mitochondria, and cytoplasm of all eukaryotes and eubacteria (Hill and Hemmingsen, 2001). Chaperonins interact with other proteins and promote their assembly into functional complexes (Roy, 1989). A decrease, rather than an increase, in the intensities of spots identified as PDI and chaperonin in our studies probably reflects the susceptibility of *B. napus* to *S. sclerotiorum*. In light of the aforementioned induction of PDI in a fungi-resistant wheat line, it is possible that this gene may have utility in engineering tolerance to this devastating fungus.

Proteasome (spot 32) and other peptidases such as metallopeptidase (spot 14) and threonine endopeptidase (spot 22; Fig. 2-3; Table 2-2) were also identified in our study. The 26S proteasome is a multicatalytic proteinase complex in eukaryotes that is comprised of a 20S core particle (CP) that functions in proteolysis and a 19S regulatory particle (RP) that recognizes the protein targeted for degradation (Suty et al., 2003). The 20S proteasome consists of four seven-membered rings formed by α and β subunits (Baumeister et al., 1998). In our study, we identified as α subunit of a proteasome-like protein as being up-regulated. A role of proteasome in cell cycle progression, senescence, and the elicitation of defense responses (Conrath et al., 1998; Ito et al., 1999) has been reported, and the possible involvement of induced modified proteasomes known as “plant defense proteasomes” in the activation of plant defense reactions has been suggested (Suty et al., 2003). The increase in the intensity of the spot

identified as a proteasome in this study further supports an important role for this protein in mediating plant responses to pathogens.

2.3.6. Antioxidant defense and detoxification

Plants possess complex defense mechanisms that respond to pathogen attack, including a rapid induction of antioxidant enzymes. A spot identified as the antioxidant enzyme POX was increased ~2-fold in intensity following *S. sclerotiorum* challenge (spot 21; Fig. 2-3; Table 2-2). Plant POX has also been associated with a number of cellular and physiological functions, including the regulation of reactive oxygen species (ROS) by H_2O_2 removal, biosynthesis and degradation of lignin in cell walls, hormone signaling, oxidation of toxic reductants, and defense against pathogens (Fossdal et al., 2001; Yoshida et al., 2003). Nevertheless, induction of POXs in host plants due to invasion of fungal pathogens has been reported in a number of studies (Curtis et al., 1997; Fossdal et al., 2001; Yoshida et al., 2003); however, the presence of multiple isozymes, a lack of substrate specificity, and the complex signaling and regulatory processes involving plant peroxidases makes it difficult to fully comprehend their precise physiological functions (Curtis et al., 1997; Yoshida et al., 2003). Overexpression of *POX* genes conferred enhanced abiotic stress tolerance in many plants (Curtis et al., 1997), and antisense suppression of the expression of POX resulted in an impaired oxidative burst and susceptibility to both fungal and bacterial pathogens (Bindschedle et al., 2006). It is therefore possible that plants respond to pathogen infection by increasing the cellular levels of antioxidant enzymes, including peroxidases.

To validate the role of POX in mediating *B. napus* responses to *S. sclerotiorum*, we characterized the expression of a *POX* gene using qRT-PCR. Oligonucleotide primers and probes were designed based on the *A. thaliana POX* sequence (due to the absence of *B. napus* sequence data); however, these primers failed to amplify the expected region of *B. napus* POX. Therefore, we evaluated the response of this *POX* gene using *A. thaliana* cDNA from various time points following *S. sclerotiorum* challenge, but the results did not show any significant changes in the abundance of this transcript (Fig. 2-5A). To further confirm a potential role for peroxidase in mediating responses to the pathogen, we performed peroxidase enzyme assays on crude protein extracts prepared from tissues of *B. napus*. Our results are presented in Figure 2-6A, and it is evident that peroxidase activity increasd sharply and significantly ($p < 0.05$) after 24 h following inoculation. POX enzyme assay results therefore support our 2-DE gel results and further illustrate the value of proteome analysis to identify proteins (consequently genes) that may be affected as a result of a stress.

Interestingly, no other proteins associated with the antioxidant defense responses of plants were identified in our 2-DE gels, a result that is different from those reported in investigations of other pathosystems. One example is SOD, which has been implicated in a number of pathosystems (Subramanian et al., 2005). To evaluate further the role of SOD in the *B. napus*/*S. sclerotiorum* pathosystem, we performed SOD enzyme assays. The results from these assays indicated that SOD activity was significantly ($p < 0.05$) reduced at 24 h, followed by a significant ($p < 0.05$) increase in the 36–48 h time frame (Fig. 2-6B). These

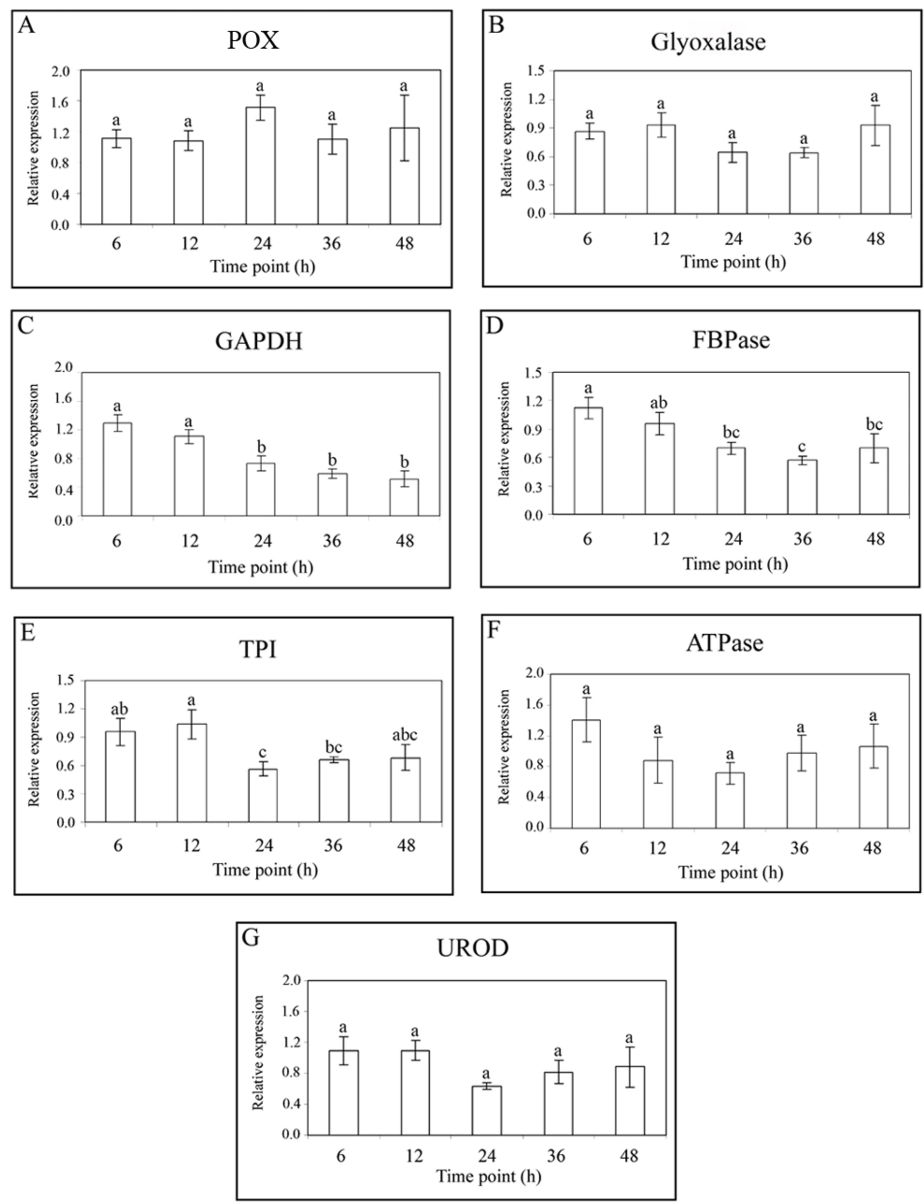


Figure 2-5. Changes in the relative abundance of transcripts of (A) POX, (B) glyoxalase, (C) GAPDH, (D) FBPase, (E) TPI, (F) ATPase, and (G) UROD at various time points after inoculation with *S. sclerotiorum*. All data were normalized against an actin endogenous control using the comparative C_T method (Livak et al., 2001), and fold changes in transcript abundance are expressed relative to the uninoculated control at the corresponding time point.

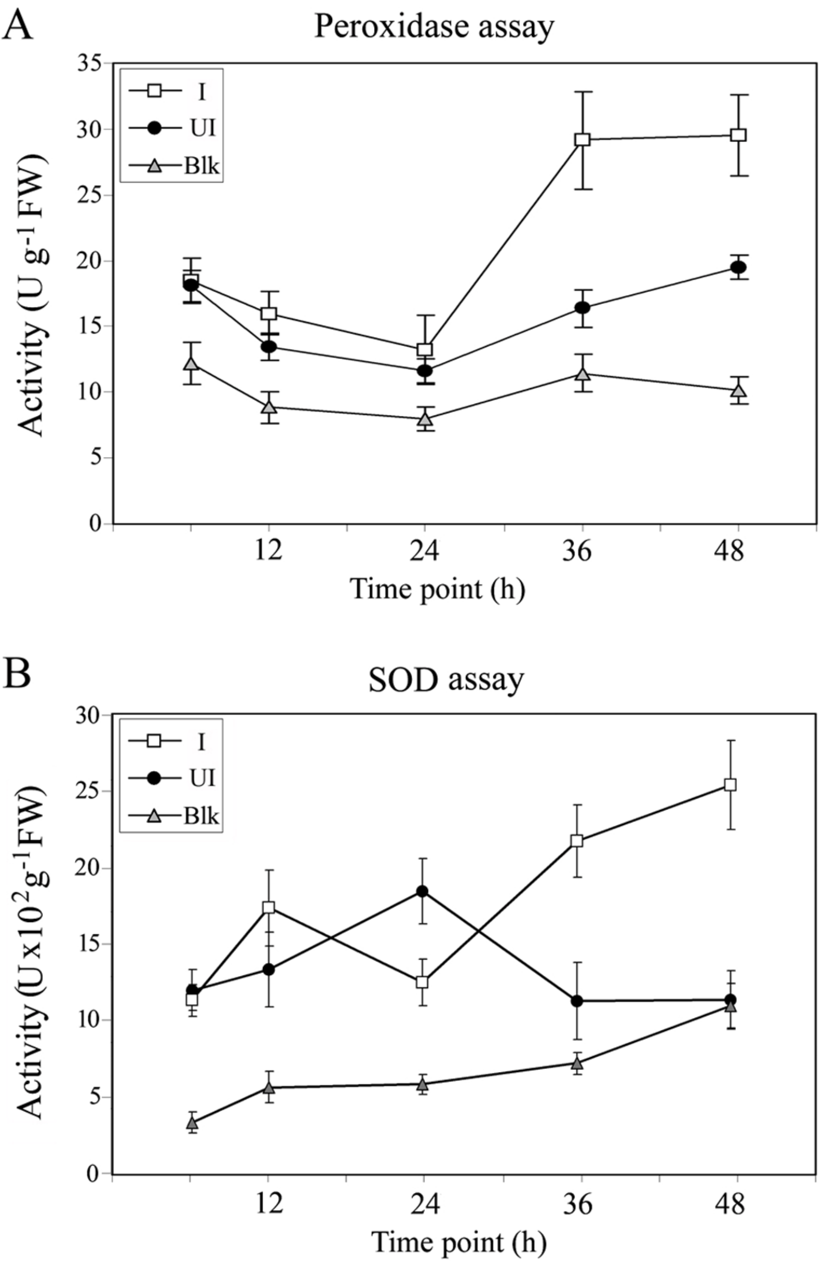


Figure 2-6. Peroxidase (A) and SOD (B) activity in *B. napus* leaves at various time points after inoculation with *S. sclerotiorum*. Proteins were extracted from leaf tissues that were inoculatd (I), uninoculated (UI), and untreated (Blk) without wounding or inoculation.

temporal changes in the activity of SOD following inoculation with the pathogen are quite similar to those observed for POX. As mentioned earlier, it was surprising that SOD was not among the spots identified in our 2-DE gels as being significantly affected by the pathogen challenge. The discrepancy between our 2-DE and enzyme assay results may be due to the fact that the observed increase in enzyme activity represents total SOD activity, whereas changes in spot intensities on 2-DE gels will be limited to the various isoforms of SOD. In other words, an individual isoform has to be elevated significantly ($p < 0.05$) for the PDQuest software to consider a spot to be significantly altered, whereas SOD enzyme assays are indicative of total activity, which appears to be significantly ($p < 0.05$; Fig. 2-6B) altered in the pathogen-challenged leaf tissues. Our results suggest that POX and SOD, as well as perhaps additional antioxidant emzymes, are elevated at 36–48 h after inoculation. However, the delayed increase in antioxidant defense responses in *B. napus* may be one of the reasons behind the successful establishment of fungal infection at earlier time points (Fig. 2-2).

Another interesting enzyme identified in this study as being increased ~3-fold at 48 h after inoculation is a putative glyoxalase, more specifically glyoxylase I (spot 25; Fig. 2-3; Table 2-2). The glyoxalase system catalyzes the detoxification of methylglyoxal (MG), a toxic byproduct generated spontaneously from dihydroxyacetone phosphate, and is made up of glyoxalase I and II (Thornalle, 1990). MG can also accumulate as a result of the leakage of a 1,2-enediolate intermediate from the active site of TPI (Richard, 1991), or as a result of MG synthase action, and can cause numerous deleterious effects including protein

degradation and modification and inactivation of antioxidant defense systems (Martins et al., 2001). It has also been reported that a TPI deficiency causes an increase in MG levels in infants (Ahmed et al., 2003), and it is tempting to speculate that the observed decrease in TPI has a role in increasing the MG levels during *S. sclerotiorum* infection of susceptible host species. Although the glyoxalase system has been studied extensively in response to abiotic stresses, to our knowledge, there are no papers describing the involvement (or lack thereof) of this system following infection of *B. napus* by *S. sclerotiorum*. However, a potential role for glyoxalase I has been suggested in the resistance of maize to *Aspergillus flavus* (Chen et al., 2004), indicating the possibility that glyoxalase plays an important role in mediating plant responses during pathogen challenge. This argument is further strengthened by the increased glyoxylase I activity observed in maize lines resistant to *A. flavus* (Chen et al., 2004).

Glyoxalases have been modulated via genetic engineering to confer salinity tolerance (Singla-Pareek et al., 2007; Veena et al., 1999); however, their utility in engineering disease resistance has not been demonstrated. As may be the case with the antioxidant enzymes, the timing of induction of glyoxalase in *B. napus* following pathogen challenge may determine the outcome of the interaction between host and pathogen (i.e., tolerance vs. susceptibility), a suggestion that must be verified through detailed genetic and molecular approaches. In this case of *S. sclerotiorum* infection of *B. napus*, glyoxalase I appears to be induced at a later stage (48 h) and may not be able to combat the deleterious effects of methylglyoxal. To further investigate the involvement of glyoxalase I in the *B.*

napus/*S. sclerotinia* pathosystem, primers specific to the *A. thaliana* glyoxalase I gene were designed, and qRT-PCR analysis performed. Our results indicated that glyoxalase I transcripts increased during the 36–48 h period, the timing of which paralleled the increase in the abundance of the protein spot identified as glyoxalase I, suggesting a potentially important role for this enzyme. The utility of overexpressing glyoxalase to engineer tolerance to *S. sclerotiorum* (and perhaps other pathogens) is currently being investigated in our laboratory.

2.3.7. Proteins involved in hormone biosynthesis and signaling

Plant hormones including jasmonic acid (JA) and ethylene play important roles in mediating plant responses to stress. Both JA and ethylene signaling pathways are essential for the induction of nonspecific disease resistance, which is distinct from the salicylic acid-regulated systemic acquired resistance (Dong, 1998). Proteins involved in both ethylene biosynthesis (such as methionine adenosyltransferase, MAT; spot 18) and JA-signaling (such as JA-responsive protein, JR1; spot 6; Fig. 2-3; Table 2-2), were identified in our study. MAT catalyzes the synthesis of the ethylene precursor *S*-adenosylmethionine (AdoMet) and plays an important role in mediating the cross-talk between ethylene and NO signaling pathways (Delledonne et al., 1998; Lindermayr et al., 2006). For example, AdoMet is a substrate for ethylene and polyamine biosynthesis, and it has been suggested that NO may regulate ethylene biosynthesis by inhibiting MAT activity (Wang et al., 2006). JA has crucial roles in regulating many plant processes including mediating resistance to pathogens (Creelman and Mullet, 1997). JA-responsive (*JR*) genes, including *JR1*, have been demonstrated to be

induced by wounding (León et al., 1998). Interestingly, a recent microarray investigation into the transcriptional changes in *B. napus* following *S. sclerotiorum* challenge conducted in our laboratory revealed an increase in the transcript abundance of many JA biosynthesis-related proteins (Yang et al., 2007), which further supports an important role for JA in this pathosystem.

2.3.8. Validation of transcript abundance for selected genes

In addition to previously described gene expression analysis of peroxidase (Fig. 2-5A) and glyoxalsae (Fig. 2-5B), we also investigated the relative abundance of transcripts for GAPDH, FBPase, TPI, ATPase and UROD. For all of the genes, the primers and probes were designed based on available *A. thaliana* sequence information (Table 2-1), but we were unsuccessful in using these primers to amplify the *B. napus* homologues of these genes (in all cases except ATPase) in the reverse transcriptase PCR (RT-PCR) experiments (data not shown). Because both *B. napus* and the model plant *A. thaliana* are closely related and both are susceptible to *S. sclerotiorum*, we used *A. thaliana* cDNA from various time points after infection with *S. sclerotiorum* to perform the qRT-PCR analysis. Our qRT-PCR results indicated that GAPDH (Fig. 2-5C), FBPase (Fig. 2-5D) and TPI (Fig. 2-5E) exhibited significant ($p < 0.05$) changes in transcript abundance following pathogen challenge. Furthermore, the changes in transcript abundance for GAPDH and FBPase were similar to those observed for their corresponding protein spots on 2-DE gels at the time points investigated. Contrary to these results, transcriptional analysis revealed no significant ($p < 0.05$) change for ATPase (Fig. 2-5F) and UROD (Fig. 2-5G). Even though the

gene expression patterns for these two genes do not correlate with proteome level changes, a role for these enzymes during the response of *B. napus* to *S. sclerotiorum* cannot be ruled out due to the generally poor correlation between the transcriptome and the proteome (Gygi et al., 1999; Greenbaum et al., 2003). Our 2-DE and qRT-PCR results suggest that a decrease in three Calvin cycle enzymes, GAPDH, TPI and FBPase, may be occurring as a result of pathogen challenge which may affect the metabolism of the pathogen-challenged plant and may have implications for the eventual outcome of the infection process. In fact, as mentioned earlier, a decrease in TPI has the potential to lead to increased MG levels, which will have deleterious consequences for the plant cell.

Global gene expression analysis using microarrays and protein analysis using proteomics are promising techniques to investigate the molecular events occurring during host–pathogen interactions. Our proteomics-based investigation of the *B. napus*/*S. sclerotiorum* pathosystem revealed the identities of many proteins that were up- or down-regulated after pathogen challenge. This is the first proteomics-based investigation of this pathosystem, and the proteins identified as increased or decreased included metabolic enzymes, as well as proteins associated with protein folding, hormone signaling, and the antioxidant defense response. Furthermore, several of the proteins identified were unnamed with unknown functions. The proteomics results were validated by conducting qRT-PCR of selected genes and enzymatic activity assays for peroxidases and superoxide dismutase. Future studies will be aimed at the investigation of a link between TPI

and MG detoxifying glyoxalases, which has been suggested previously (Singla-Pareek et al., 2007) and may have utility in engineering tolerance to this pathogen.

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3. Oxalic acid-mediated stress responses in *Brassica napus* L.*

3.1. Introduction

Oxalic acid (OA) is ubiquitous in nature and plays diverse roles in various organisms including animals, plants, and fungi (Hodgkinson, 1977). It is secreted by many fungal species including *Postia placenta* (Micales, 1994), *Fomitopsis palustris* (Munir et al., 2001), *Wolfiporia cocos* (Causenl and Green, 2003), *Botrytis cinerea* (Gentile, 1954), *Sclerotinia sclerotiorum* (de Bary, 1886; Maxwell and Lumsden, 1970) and performs many activities related to both mycological physiology and ecology (Dutton and Evans, 1996).

Sclerotinia sclerotiorum is a necrotrophic fungal pathogen capable of infecting a broad range of hosts, including many economically-significant crops such as sunflower, soybean, and canola (Boland and Hall, 1994). Sclerotinia stem rot is considered a destructive disease, which affects the quality and yield of oilseed crops worldwide. OA secreted by *S. sclerotiorum* is generally believed to help initiate the infection and facilitate disease progression (Godoy et al., 1990; Bolton et al., 2006). The accumulation of OA has been to shown to be positively correlated with disease severity (Maxwell and Lumsden, 1970).

Evidence in the literature suggests that one of the roles of OA during *S. sclerotiorum* infection is the chelation of Ca^{2+} in the apoplast to form insoluble oxalate crystals that subsequently adversely affect the integrity of the plant cell wall (Bateman and Beer, 1965). In addition, the direct acidification within the middle lamella also enhances the activities of many cell wall degrading enzymes

*A version of this chapter has been published. Liang, Y., Strelkov, S.E., Kav, N.N.V. 2009. Proteomics 9, 3156-3173.

including polygalacturonases (Bateman and Beer, 1965; Riou et al., 1991). The secretion of OA may help to inactivate plant polygalacturonase-inhibiting proteins, thereby allowing the pathogen to overcome this specific host defense response (Favaron et al., 2004). Host polyphenol metabolism is also affected by OA, through the inhibition of polyphenoloxidase activity (Magro et al., 1984; Ferrar and Walker, 1993).

The importance of OA in pathogenicity is demonstrated by the fact that OA-deficient mutants of *S. sclerotiorum* failed to initiate infection despite the production of equal or even higher quantities of cell wall degrading enzymes (Godoy et al., 1990; Lumsden, 1979). In addition to facilitating infection by *S. sclerotiorum*, OA appears to enter the vascular stream, causing wilting of the foliage (Noyes et al., 1981). The wilting results from the disruption of abscisic acid (ABA)-induced stomatal closure by OA, which may enable further growth of the fungus beyond the initially infected tissues (Guimarães and Stotz, 2004). The utility of oxalate oxidase to combat the OA secreted by the fungus as a potential strategy for crop improvement has also been previously reported (Thompson et al., 1995; Dong et al., 2008; Chipps et al., 2005; Livingstone et al., 2005). OA has been shown to suppress the oxidative burst of host plants through a reduction in the production of reactive oxygen species (ROS), and by interfering with ROS-mediated signaling pathways (Cessna et al., 2000). Secretion of OA by *S. sclerotiorum* has also been suggested to trigger programmed cell death (PCD) by induction of ROS as pathogen invasion progresses (Kim et al., 2008). Although the results seem to contradict each other, it is possible that the effect of OA might

depend on temporal effects, whereby suppression of ROS production occurs during establishment of infection and the induction of ROS leading to PCD and necrosis occurs during subsequent stages of infection, which helps to promote the compatible interaction between the pathogen and its host (Kim et al., 2008).

In order to further characterize the various effects elicited by OA in the host plant, we performed a detailed analysis of the proteome-level changes and oxidative/signaling responses that occur in *Brassica napus* L. in response to exogenous treatment with OA.

3.2. Materials and methods

3.2.1. Fungal materials and OA treatment

In order to determine the optimum concentration of OA to be used in the experiments, liquid cultures of *S. sclerotiorum* and *B. cinerea* were grown and the pH of the medium was determined. Briefly, a 5 mm diameter plug was excised from the periphery of a 3-day-old fungal colony grown on potato dextrose agar (PDA; Becton Dickinson; Franklin Lakes, NJ, USA) and used to inoculate 100 mL minimal salts medium containing 1% w/v pectin (Yajima and Kav, 2006). A plug of PDA without mycelia was used to inoculate control medium. The cultures were agitated at ~100 rpm for 5 days at room temperature. Mycelia were harvested by centrifugation at 15 300 × *g* for 20 min at 20°C and vacuum filtration (0.22 μm) after which the pH of the medium was determined. Three biological replicates were independently performed for each pathogen (*S. sclerotiorum* or *B. cinerea*) and control.

Various concentrations of OA (20, 40, 100, 200 mM) were prepared with or without adjustment to the pH value of the media tested above (Bateman and Beer, 1965; Favaron et al., 2004; Dong et al., 2008). A 5 µL volume of hydrochloric acid (20, 40, 100 or 200 mM) and water were used as a control, and the severity of necrosis was used to determine the appropriate concentration of OA and pH for subsequent experiments.

3.2.2. Treatment with OA and inoculation with *S. sclerotiorum*

For proteome analysis, enzyme activity assays and real-time PCR, 18-day-old *B. napus* cv. Westar seedlings with two true leaves, which were grown at 22°C day/18°C night with a 18h photoperiod, were placed in a humidity chamber for 24 h prior to treatment. Leaves were wounded by gently scratching them with a pipette tip and were either treated with OA (treatment with water as control) or inoculated with a 5 mm agar plug containing 3-day-old *S. sclerotiorum* mycelium (agar plug without mycelia as control). Plants were returned to the humidity chamber for an additional 24 h to prevent rapid evaporation of the OA solution and to keep treatment conditions consistent with those used in a previous study (Liang et al., 2008). Plants were then transferred to the greenhouse for further growth under the conditions described above. Whole leaves (2 true leaves from each plant) from ten treated/inoculated and control plants were collected (at 0.5, 1, 3, 6, 12, 24, 36, and 48 h for OA treatment and 6, 12, 24, 36, and 48 h for *S. sclerotiorum* inoculation) and stored at –80°C until used. Three biological replicates were independently performed.

3.2.3. Extraction of protein

Proteins from collected tissues were extracted as described earlier (Liang et al., 2008). Briefly, harvested leaves were pooled and 0.3 g of tissue was finely ground in liquid nitrogen and then suspended in 1 mL of actone containing 10% w/v trichloroacetic acid and 0.07% w/v DTT. After incubation at –20°C for 1 h, the suspension was centrifuged (18 000 × *g* for 15 min at 4ºC) and the pellet was washed five times with 1 mL of ice-cold acetone containing 0.07% w/v DTT. The pellet was dried under vacuum and the proteins were resolubilized in 400 μL rehydration/sample buffer (Bio-Rad, Hercules, CA, USA) containing 1.0% v/v tributylphosphine (Bio-Rad) and incubated overnight at 4ºC. The samples were subsequently centrifuged (18 000 × *g* for 15 min at 4ºC) and the supernatants were stored at –20°C until electrophoresis. Protein extraction was performed twice from each of three independent biological replicates for each time point (i.e., six independent extracts per time point). The protein concentration was determined using protein assay dye reagent (Bio-Rad) and BSA as a standard (Liang et al., 2008).

3.2.4. 2-DE and image analysis

IEF in the first dimension and separation by SDS-PAGE in the second dimension were performed as described previously (Liang et al., 2008). IPG strips (17 cm; pH 4–7; Bio-Rad) were passively rehydrated overnight with 300 μL rehydration/sample buffer containing the extracted proteins (400 μg). IEF was performed on a PROTEAN IEF cell (Bio-Rad) with the settings: 250 V for 15 min, linear increase to 10 000V over 3 h, focused for 60 000 Vh, and held at 500 V for 1 h. Focused IPG strips were equilibrated in 5 mL equilibration buffer-I (6

M urea, 2% SDS, 0.37 M Tris-HCl, pH 8.8, 20% glycerol, and 130 mM DTT) twice (10 min each) and then incubated in equilibration buffer-II (6 M urea, 2% SDS, 0.37 M Tris-HCl, pH 8.8, 20% glycerol, and 135 mM iodoacetamide) twice (10 min each). The equilibrated strips were placed on top of 13% polyacrylamide gels and second dimension SDS-PAGE was performed in a PROTEAN II xi Cell (Bio-Rad) with the Precision Plus Protein Standard (Bio-Rad). After electrophoresis, the gels were stained with a Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. The sensitivity of this staining kit is < 10 ng and is linear up to 10 000 ng.

2-DE gel images were recorded using a GS-800 calibrated densitometer (Bio-Rad) and a total of 12 gel images (gels from two technical replicates of three biological replicates for both treatment and control) for each time point were assembled in a matchset using PDQuest software version 7.3.1 (Bio-Rad). Automated spot detection was performed with software and then manually verified. The intensities of each matched spot were analyzed and significantly ($p < 0.05$) altered spots were identified with the Student's *t*-test feature of the software. To verify the results generated by the PDQuest software, spot quantification values were exported and statistical analysis was performed as described in the following section. All protein spots that were reproducibly and significantly different in abundance between the treatment and control were chosen for analysis by MS/MS, irrespective of the fold changes observed. The expression ratios for each spot at each time point were determined as a ratio of

spot intensities in the treatment gels to the corresponding intensities in the control gels, in order to perform comparisons of protein intensity changes over time.

3.2.5. MS analysis

The protein spots were excised and an automated in-gel digestion was performed on a Mass Prep Station (Micromass, Manchester, UK). In-gel tryptic digests were analyzed by LC-MS/MS using an Apex-Qe 9.4T FT-ICR (Bruker Daltonics Inc., Billerica, MA, USA). LC separations were performed using an Agilent 1100 Capillary HPLC system operated at 10 μL /min with a pre-column split producing a flow rate of 400 nL/min onto a 75 μm × 10 cm, 5 μm particle size, C18 reversed phase column (Michrom Bioresources). Peptides were eluted using a linear 2% B/min gradient starting at 95% A (A = 0.2% formic acid in water, B = 0.2% formic acid in ACN). Mass measurements were taken from *m*/*z* 350–1600 and data-dependent MS/MS was performed on doubly and triply charged precursors. A cell fill time of 0.5 s was used for MS measurements and 1 s for MS/MS. Data files were processed using Apex Data Analysis software generating a Mascot generic file (mgf) that was then searched using the Mascot version 2.1 search engine (Matrix science, MA, USA). The mgf peak lists were generated using Mascot Distiller 2.1.1.0, which consist of the precursor mass and its associated fragment ions using the following parameters. Uncentroiding of both MS and MS/MS data used a peak half height of 0.2 and 20 data points per Da. Peak picking for both MS and MS/MS data used a minimum *S*/*N* of 2, minimum peak width of 0.01, and a maximum peak width of 1. The mgf were used to search the NCBI non-redundant database using an in-house copy of

Mascot using the following search criteria: one missed cleavage, 0.6 *m/z* MS/MS tolerance, including oxidized methionine variable modification and carbamidomethyl cysteine fixed modification. MS/MS analysis was performed at the Institute for Biomolecular Design (University of Alberta).

3.2.6. Measurements of ROS

Measurements of ROS accumulation in response to OA treatment were performed as described by Cessna et al. (2006) with minor modifications. Leaves from 18-day-old *B. napus* plants were wounded by gently scratching with a pipette tip at four sites per leaf. The leaves were treated with OA (40mM, pH 2.5) and *S. sclerotiorum* with respective controls (water and agar plug without mycelia) at the wound sites. The accumulation of ROS was determined at selected time points (at 0.5, 1, 3, 6, 12, 24, 36, and 48 h) after treatment. Briefly, the stain solution containing 1 mM NBT and 5 mM MES was adjusted to pH 5.5 and applied on the surface of treated leaves for 10 min, and then washed in 95% v/v ethanol overnight to remove chlorophyll in order to increase the contrast of the dark blue ethanol insoluble NBT stain against the background. The accumulation of the oxidant in the treated leaves was indicated by the presence of insoluble NBT. The experiment was independently performed three times.

3.2.7. Enzyme activity assays

Peroxidase (POX) and superoxide dismutase (SOD) assays were performed as previously described (Liang et al., 2008). Catalase (CAT) activity was assayed as described by Khanam (2005) with some minor modifications. The harvested leaves (100 mg) described above were pooled and homogenized in 1 mL of 16.6

mM sodium phosphate buffer (pH 7.2) containing 0.6 mM EDTA and 50 mg polyvinyl-polypyrrolidone (PVP) and centrifuged at 20 400 × g, 15 min, and 4°C. Twenty μL of the resulting supernatant (tissue extract) were added to 180 μL buffer (50 mM sodium phosphate, pH 7.2, 1 mM EDTA, and 50 mM H_2O_2) and the reaction solution incubated for 10 min. The decrease in absorbance at 240 nm was measured and used to calculate enzyme activity, with one unit defined as the conversion of one μmol of H_2O_2 per minute (Dhindsa et al., 1981). Enzyme activities were expressed as specific activity (units mg^{-1} protein) and then relative specific activities (treatment/control) were calculated for all time points following treatment. All enzyme assays were repeated twice with tissue extracts from three independent biological replicates.

3.2.8. Real-time PCR analysis

Total RNA was extracted from leaves (100 mg) using an RNeasy Plant Mini Kit (Qiagen, ON, CA) and treated with Rnase-free Dnase (Qiagen) to remove any contaminating genomic DNA. First strand cDNA was synthesized as per the manufacturer's instructions from 50 ng total RNA using an iScript cDNA Synthesis Kit (Bio-Rad). The real-time PCR was performed using the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a total reaction volume of 10 μL containing 2.5 μL of 10 × diluted cDNA template, 2.5 μL primer mixture (3.2 μM each primer), and 5.0 μL SYBR Green Master Mix [0.8% v/v glycerol, 2% v/v DMSO, 50 mM KCl, 3mM $MgCl_2$, 0.01% v/v Tween 20, Tris pH 8.3, 0.2 mM dNTPs, 1 × ROX, 0.25 × SYBR Platinum Taq 0.03 units/μL]. Primers were designed using Primer Express Software version 3.0

(Applied Biosystems). The primers used for each gene and *actin* as the endogenous reference gene are listed (Table 3-1). Relative gene expression was evaluated according to the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001).

3.2.9. Statistical Analysis

The data were analyzed using the generalized linear model procedure for ANOVA followed by Duncan's multiple range test ($p < 0.05$) to identify differences among time-points. The Student's *t*-test was used to identify significant ($p < 0.05$) changes at specific time-points. All analyses were conducted using SAS software (version 9.1, SAS Institute, Cary, NC, USA).

3.3. Results

3.3.1. Determination of optimum OA treatments

The pH of cultures of *S. sclerotiorum* and *B. cinerea* was measured to determine the optimal pH to be used for OA treatment of *B. napus* leaves. The pH of both culture media was approximately 2.5, which was significantly ($p < 0.05$) lower than that of the non-inoculated medium (pH ~3.0). The observed morphological changes caused by different concentrations of HCl and OA with and without adjustment of the pH on *B. napus* leaves after 24 h indicate that the application of 40 mM OA (pH 1.8) on leaf tissue resulted in necrosis with larger lesions correlated to increasing concentrations of OA. Treatment with HCl

Table 3-1. Primer sequences used for real-time PCR

Gene	Accession no.	Primers	
		Forward	Reverse
NPR1	AF527176	5’-TGCTCTGTTGATCGCGAA AC-3’	5’-CGCCTTTGGCAGCTAACTTC-3’
TGA5	EE463024	5’-CAGACGCTGGCAGGAAGATAA-3’	5’-GCTCAGGTTCACTCGCATGA-3’
TGA6	CN732338	5’-CAGCCAAGAATGATGTCTTCCA-3’	5’-CCCACCAAGCCACAAGAAAC-3’
PDF1.2	AY884023	5’-AGTGGGACATGGTCAGGAGTCT-3’	5’-CCATGTTGTGCTCCTTCAAGTC-3’
AOC	CD828070	5’-CAAGCAAAAACCCGAGGAGTT-3’	5’-CTGGTGGCATATTGACTCGAAA-3’
EIN2	EE477530	5’-GGAAAATGCACAACAGCAGTCA-3’	5’-GTCCGGCCTTTCCTACAAGAG-3’
ERF2	CN737061	5’-GGGTTTGGTTAGGGACGTTTG-3’	5’-GGAACCACGCATCCTAAAAGC-3’
ERF4	EE462058	5’-TGGCGCGTGTTACCAGATC-3’	5’-GACCACGACCTCCGTTTCC-3’
ABI5	DY003521	5’-GGACCGTTAAGCCCGGTATC-3’	5’-TCCTTCCCCTTAACCCTCTCA-3’
OXO	AAX35339	5’-TTCTTGACTTTGCGCTATTTGC-3’	5’-GACTGTAGCAGGCGGTAGGAA-3’
NOX	EV035965	5’-TCTTGGCATTGGTGCAACTC-3’	5’-CCGCTTGCTCCTCCATTTTA-3’
Actin	AF111812	5’-ACGAGCTACCTGACGGACAAG-3’	5’-GAGCGACGGCTGGAAGAGTA-3’

(20mM, pH 1.8) produced smaller lesions than those induced by OA (pH 1.8). Necrosis was also clearly visible when 40 mM OA (pH 2.5) was applied to the wound sites, but no concentrations of HCl (pH 2.5) produced necrosis. These observations suggest that the necrosis caused by OA at this pH is not merely a result of acidity, but may also reflect additional OA-specific effects. Based on these results, a concentration of 40 mM OA, pH 2.5 was chosen for all experiments. The effects of treatment of *B. napus* leaves with OA (40 mM, pH 2.5) over time are illustrated (Fig. 3-1). Necrosis could be observed as early as 1 h after treatment and continued to progress up to 48 h.

3.3.2. Identification and classification of the differentially expressed proteins

The proteome-level changes induced by OA treatment were examined at different times, with proteins separated using 2-DE as illustrated in Figure 3-2. A total of about 300–400 protein spots were resolved on the gels at each time-point. Protein spots whose intensities were observed to be reproducibly and significantly ($p < 0.05$) altered as a result of OA treatment, as indicated by the PDQuest software followed by statistical analysis with SAS software, were selected for MS/MS analysis. A total of 32 proteins were identified with five spots (4, 6, 11, 20, and 22) showing significant differences in abundance at more than one time point, for a grand total of 37 individual spots exhibiting significant changes in response to OA stress. The respective number of up- and down-regulated protein spots was 13 and 24 (Fig. 3-3A).

Most spots exhibited more than a 1.5-fold change in abundance, with 22 exhibiting changes greater than a twofold up- or down-regulation (fold > 2 or <

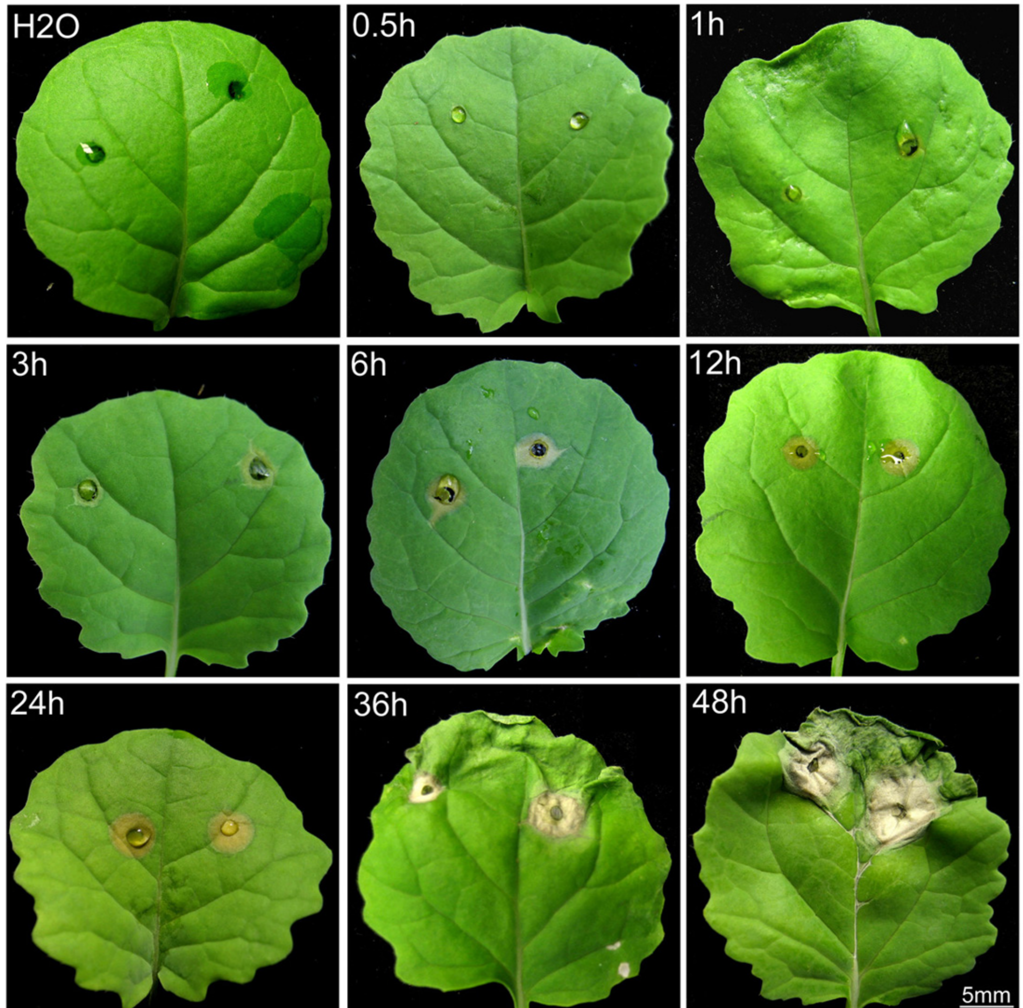

Figure 3-1. Effect of leaf treatment with oxalic acid. The H_2O-treated leaves as control appeared identical at all time points, so only a representative leaf is shown. All other leaves were treated with OA (40 mM, pH 2.5) and then observed at each time point as indicated in the figure.

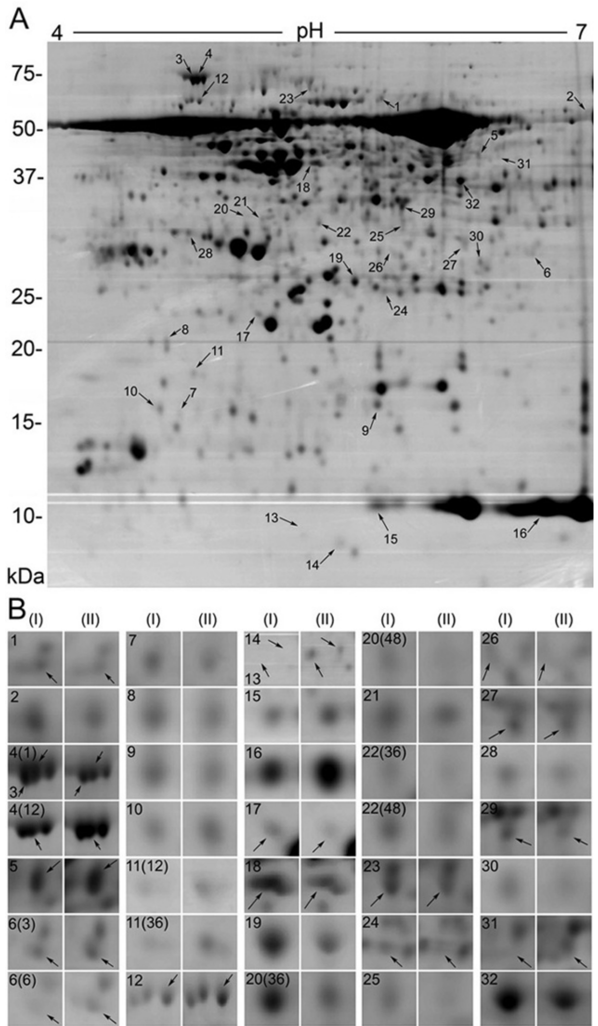

Figure 3-2. Proteome-level changes in leaves of *B. napus* induced by treatment with OA. (A) Representative image of 2-DE gel was performed by IEF/SDS-PAGE separation followed by staining with colloidal Coomassie Blue. Significantly altered spots are indicated by numbers and arrows and their identities are detailed in Table 2. (B) Closer view of protein spots demonstrating changes in intensity. Numbers within brackets correspond to the specific time points at which changes in intensity were observed and only apply to those with changes at more than one time point. The spots from H_2O (I) and OA (II) treatment are shown in these panels.

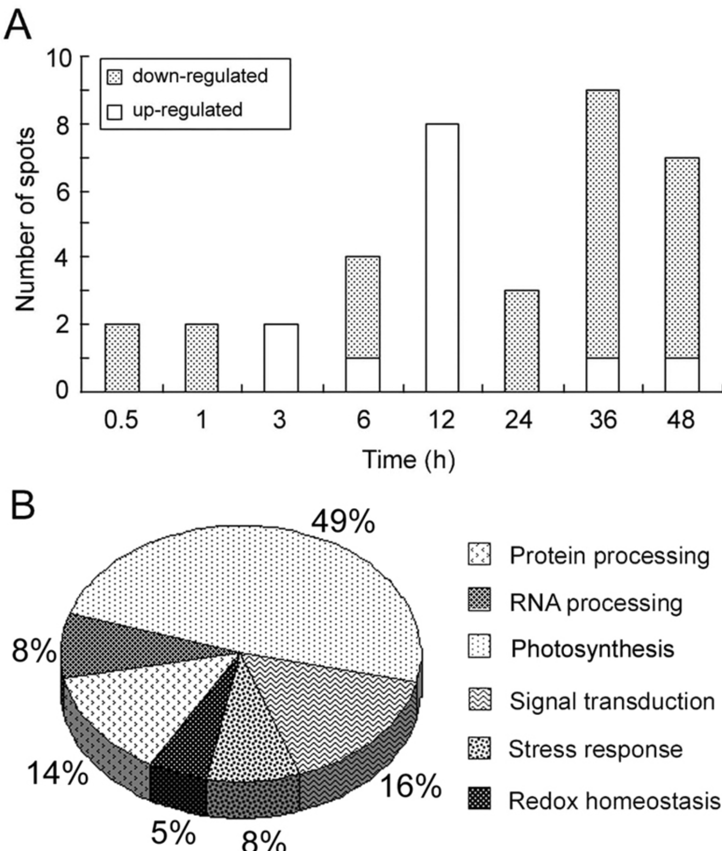

Figure 3-3. Temporal distribution of spots with altered intensity and their functional classification. Distribution of spots whose intensities were significantly affected by OA treatment at each time point (A). Each bar illustrates number of proteins with an increase in spot intensity (shaded) and decrease in spot intensity (unshaded). The functional classification of the identified proteins is shown (B).

Table 3-2. List of the differentially expressed proteins in *B. napus* identified by MS/MS analysis which respond to oxalic acid.

Spot[a]	Accession[b]	Protein name	Expression ratio[c]	Score[d]	C[e]	MW (kDa)/pI Theor.	MW (kDa)/pI Obs.	Sequence[f]
1	21536501	Phosphoglycerate dehydrogenase-like protein [*Arabidopsis thaliana*]		130/55	5	63.6/ 6.32	62.7/ 5.88	K.ISLCDALIVR.S R.GGVIDEDALVR.A R.LAVQLVAGGSGVK.N
2	15221044	dihydrolipoamide dehydrogenase 1, mitochondrial / lipoamide dehydrogenase 1 (MTLPD1) [*Arabidopsis thaliana*]		348/54	16	54.2/ 6.96	57.2/ 6.91	R.GALGGTCLNVGCIPSK.A K.HIIVATGSDVK.S K.KIVSSTGALSLSEVPK.K K.IVSSTGALSLSEVPK.K R.TPFTSGLDLEK.I K.AEEDGVACVEFIAGK.H K.EAAMATYDKPIHI.-
3	6746592	heat shock protein 70 [*Arabidopsis thaliana*]		374/54	9	77.2/ 5.13	70.8/ 4.92	K.QFAAEEISAQVLR.K R.IINEPTAASLAYGFER.K K.DIDEVILVGGSTR.I K.SEVFSTAADGQTSVEINVLQGER.E
4	6746592	heat shock protein 70 [Arabidopsis thaliana]		373/54	9	77.2/ 5.13	71.1/ 4.92	K.QFAAEEISAQVLR.K R.IINEPTAASLAYGFER.K K.DIDEVILVGGSTR.I K.SEVFSTAADGQTSVEINVLQGER.E
5	1944432	ribulosebisphosphate carboxylase [*Arabidopsis thaliana*]		259/54	13	48.0/ 6.12	43.8/ 6.38	K.TFQGPPHGIQVER.D K.YGRPLLGCTIKPK.L R.DNGLLLHIHR.A R.ESTLGFVDLLR.D R.DLAVEGNEIIR.-
6	30959088	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Brassica juncea*]		214/52	11	53.4/ 5.88	28.87/ 6.60	K.LNYYTPEYETK.D K.DTDILAAFR.V K.TFQGPPHGIQVER.D K.DDENVNSQPFMR.W R.FLFCAEAIYK.S
7	110742393	thioredoxin m4 [*Arabidopsis thaliana*]		129/52	11	20.0/ 9.62	15.5/ 4.85	K.INTDESPNTANR.Y R.SVPTVIIFK.G
8	16033628	translationally controlled tumor protein [*Brassica oleracea*]		99/51	19	19.1/ 4.63	20.6/ 4.79	K.EIENGILWEVEGK.W R.LQEQPTYDK.K K.LTPEQQEEFK.K
9	30679426	PETC (Photosynthetic electron transfer C) [*Arabidopsis thaliana*]		153/51	12	22.8/ 8.58	16.0/ 5.86	K.GDPTYLVVENDK.T K.FLCPCHGSQYNAQGR.V
10	468773	ribosomal protein L12 [*Arabidopsis thaliana*]		90/53	12	19.7/ 5.51	15.8/ 4.75	K.IGSEISSLTLEEAR.I R.ILVDYLQDK.F
11	17813	BnD22 drought induced protein [Brassica napus]		163/45	18	23.7/ 5.89	18.1/ 4.92	K.LWAVDVSSSAAK.E K.EPAIIIGGESTAPNSLFK.I K.IEEATEANTYK.L
12	1351030	RuBisCO large subunit-binding protein subunit alpha, chloroplast precursor (60 kDa chaperonin subunit alpha) [*Brassica napus*]		162/41	5	57.7/ 4.84	60.8/ 4.95	K.DSTTLIADAASKDELQAR.I K.VGAATETELEDR.K

Table 3-2. Continued

13	17850	ribulose bisphosphate carboxylase [*Brassica napus*]	137/41	16	20.5/ 8.23	9.1/ 5.49	R.EHGSTPGYYDGR.Y K.TEYPNAFIR.I R.IIGFDNNR.Q
14	17852	ribulose bisphosphate carboxylase /oxygenase small subunit [*Brassica napus*]	276/53	23	20.5/ 8.23	8.9/ 5.67	R.EHGSTPGYYDGR.Y K.LPLFGCTDSAQVLK.E K.TEYPNAFIR.I R.IIGFDNNR.Q
15	79013990	chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit precursor [*Brassica napus*]	273/53	27	20.5/ 8.23	10.4/ 5.86	K.EVDYLLR.N R.EHGSTPGYYDGR.Y K.LPLFGCTDSAQVLK.E K.TEYPNAFIR.I R.IIGFDNNR.Q
16	17852	ribulose bisphosphate carboxylase /oxygenase small subunit [*Brassica napus*]	279/53	27	20.5/ 8.23	10.2/ 6.72	K.EVDYLLR.N R.EHGSTPGYYDGR.Y K.LPLFGCTDSAQVLK.E K.TEYPNAFIR.I R.IIGFDNNR.Q
17	21133	oxygen-evolving complex of photosystem II [*Sinapis alba*]	77/50	11	28.1/ 6.84	23.1/ 5.26	R.EVEYPGQVLR.Y R.TADGDEGGKHQLITATVNGGK.L
18	30687999	RCA (RUBISCO ACTIVASE) [*Arabidopsis thaliana*]	341/50	18	48.8/ 7.55	41.2/ 5.55	R.GLAYDTSDDQQDITR.G K.VPLILGIWGGK.G K.SFQCELVMAK.M K.MGINPIMMSAGELESGNAGEPAK.L K.MCCLFINDLDAGAGR.M R.VYDDEVR.K R.VYDDEVRK.F
19	742408	triose phosphate isomerase [*Arabidopsis thaliana*]	221/50	23	27.4/ 5.24	26.1/ 5.75	K.FFVGGNWK.C K.VAYALAQGLK.V R.EAGSTMDVVAAQTK.A K.VASPAQAQEVHDELRK.W K.NVSADVAATTR.I
20	15239735	THI1 (THIAZOLE REQUIRING) [*Arabidopsis thaliana*]	177/47	14	36.8/ 5.82	32.9/ 5.19	K.HAALFTSTIMSK.L K.LFNAVAAEDLIVK.G K.ALDMNTAEDAIVR.L R.MGPTFGAMMISGQK.A
21	15239735	THI1 (THIAZOLE REQUIRING) [*Arabidopsis thaliana*]	191/48	20	36.8/ 5.82	32.7/ 5.27	K.HAALFTSTIMSK.L K.LFNAVAAEDLIVK.G K.IVVSSCGHDGPFGATGVK.R K.ALDMNTAEDAIVR.L R.MGPTFGAMMISGQK.A
22	15239735	THI1 (THIAZOLE REQUIRING) [*Arabidopsis thaliana*]	320/48	26	36.8/ 5.82	32.0/ 5.56	K.HAALFTSTIMSK.L K.LFNAVAAEDLIVK.G K.IVVSSCGHDGPFGATGVK.R K.ALDMNTAEDAIVR.L R.EVVPGMIVTGMEVAEIDGAPR.M R.MGPTFGAMMISGQK.A
23	42561751	FTSH8 (FtsH protease 8); ATP-dependent peptidase/ ATPase/ metallopeptidase/ zinc ion binding [*Arabidopsis thaliana*]	135/55	3	73.3/ 5.72	66.9/ 5.52	R.ADILDSALLRPGR.F R.AILSEFTEIPPENR.V
24	145322866	CA1 (CARBONIC ANHYDRASE 1); carbonate dehydratase/ zinc	336/48	26	28.5/ 5.29	25.4/ 5.91	K.EKYETNPALYGELAK.G K.YETNPALYGELAK.G K.YMVFACSDSR.V R.NIANMVPPFDK.V K.VENIVVIGHSACGGIK.G

Table 3-2. Continued

		ion binding [*Arabidopsis thaliana*]						K.VISELGDSAFEDQCGR.C
25	72256517	pyridoxine biosynthesis protein [*Lotus corniculatus* var. japonicus]		292/48	19	33.3/ 6.12	32.4/ 5.99	R.IAEEAGACAVMALER.V K.QAVTIPVMAK.A R.IPFVCGCR.N R.TKGEAGTGNIVEAVR.H R.NMDDDEVFTFAK.K
26	1346967	Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit) [*Brassica oleracea*]		211/47	8	53.4/ 5.88	28.8/ 5.95	R.DNGLLLHIHR.A R.ESTLGFVDLLR.D R.DLAVEGNEIIR.E K.EITFNFPTIDK.L
27	7525041	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Arabidopsis thaliana*]		88/55	4	53.4/ 5.88	30.0/ 6.32	R.DNGLLLHIHR.A R.ESTLGFVDLLR.D
28	681904	RNA-binding protein cp29 [*Arabidopsis thaliana*]		86/55	7	34.6/ 5.23	31.1/ 4.90	K.AINSLNGADLDGR.Q R.VSEAEARPPR.G
29	6899947	O-acetylserine (thiol) lyase [*Arabidopsis thaliana*]		128/55	5	41.5/ 6.96	33.9/ 5.99	K.IQGIGAGFIPK.N K.LIAVVFPSFGER.Y
30	21554102	chloroplast drought-induced stress protein, putative [*Arabidopsis thaliana*]		81/55	5	34.0/ 8.46	28.1/ 6.39	K.LIVLDVGLK.H K.GELIGEILR.Y
31	21634067	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Breweria rotundifolia*]		63/55	5	47.8/ 6.97	42.5/ 6.48	K.TFQGPPHGIQVER.D R.DNGLLLHNHR.A
32	15229384	mRNA-binding protein, putative [*Arabidopsis thaliana*]		56/43	5	44.1/ 8.54	37.1/ 6.29	K.DLDTVRPVVDWAK.S R.NMHFYAEPR.A

[a] Spot number as given on the 2-D gel image.

[b] gi number from NCBI database of match protein.

[c] Relative expression of identified proteins represents the average ratio (treatment/control) in spot indensity at various time points (From left to right is 0.5, 1, 3, 6, 12, 24, 36, 48 h). Asterisks (*) indicates statistically significant expression ratio from 1 at a specific time point ($p < 0.05$) by the Student's *t*-test. Significant differences as determined by ANOVA ($p < 0.05$) observed at

various time points during the entire course of the experiment are indicated by different letters.

[d] Mascot score/Mascot threshold score for each identified protein.

[e] Percent sequence coverage (%).

[f] The sequence of matched peptides

0.5; Table 3-2). The results of these analyses are summarized and the temporal changes occurring throughout the time course for each protein with significantly altered expression are presented in Table 3-2. As mentioned previously, only five proteins (spots 4, 6, 11, 20, and 22) exhibited differential abundance at more than one time point, with spots 6 and 11 up-regulated and spots 20 and 22 down-regulated. Spot 4 was up-regulated at 1 h and down-regulated at 12 h after OA treatment. All other identified proteins were observed to be either up- or down-regulated only at one time-point (Table 3-2).

Based on the available literature and the protein database Pfam (http://pfam.sanger.ac.uk) or InterPro (http://www.ebi.ac.uk/interpro) (Apweiler et al., 2001), the identified proteins could be categorized into several groups including protein processing (14%), RNA processing (8%), photosynthesis (49%), signal transduction (16%), stress response (8%), and redox homeostasis (5%) (Fig. 3-3B). The proteins involved in protein processing, signal transduction, and redox homeostasis were significantly down-regulated at all time points, whilst proteins classified in other groups were either up-regulated or down-regulated. Proteins related to RNA processing were found to be up-regulated at an earlier time-point (12 h), but down-regulated at a later time point (48 h; Table 3-2).

Some of these identified proteins were classified as photosynthesis-related, including the Rubisco large subunit (spots 6, 26, and 27), the Rubisco small subunit (spots 13, 14, 15, and 16), and the oxygen-evolving complex of photosystem II (spot 17). We observed possible degradation of proteins involved in photosynthesis (e.g., Rubisco large subunit: spots 6, 26, 27). Furthermore,

other photosynthetic proteins that were observed to be degraded include the oxygen-evolving complex of photosystem II (spot 17) and the Rubisco small subunit (spots 13, 14, 15, and 16). This may reflect the fact that OA stress can cause greater degradation of proteins involved in photosynthesis. Alternatively, it may simply reflect regular turnover of these highly abundant proteins. Incidentally, degradation of photosynthetic proteins like the Rubisco large subunit has also been observed in other proteome-level analyses of plant response to stresses (Yan et al., 2006; Taylor et al., 2005).

Other proteins identified included an important molecular chaperone, HSP 70 (spot 3, 4), which was observed to be down-regulated early upon imposition of OA stress and up-regulated at 3 h. Several proteins involved in plant stress response signaling were also identified in the current study (Table 3-2).

Among the proteins belonging to the signal transduction category, pyridoxine biosynthesis protein (spot 25), which is an ethylene (ET)-inducible protein (Sivasubramaniam et al., 1995), and thiazole requiring (THI1, spot 20, 21, 22) protein, which is identical to the thiazole biosynthetic enzyme in eukaryotes (Godoi et al., 2006), were identified as down-regulated. Interestingly, we observed all proteins involved in signal transduction to be down-regulated at later time points.

3.3.3. Oxidative stress responses

Because oxidative stress responses are an integral part of the plant defense response against pathogens, we investigated the effects of OA and *S. sclerotiorum* on the production of H_2O_2, as well as on the activities of enzymes involved in

ROS production and scavenging. We found that OA appeared to inhibit wounding-induced accumulation of H_2O_2, as evidenced by the absence of dark blue insoluble deposits of oxidized NBT around the wound site (0.5 h to 6 h; Fig. 3-4), but this deposition increased 12 h after treatment (Fig. 3-4). However, in the case of *S. sclerotiorum* challenge, we observed no differences in NBT deposition from 0.5 to 6 h post-infection compared to controls. At 12 h after pathogen challenge, we observed the absence of NBT deposition around the wound site, which incidentally coincided with the first appearance of disease symptoms (Fig. 3-4). The results suggest that while OA by itself appears to suppress H_2O_2 accumulation very early, and after an increase in H_2O_2 accumulation at 12 h post-treatment, there appears to be no obvious increase in NBT deposition at later time points. However, in the case of the pathogen, a much different effect is observed with the accumulation of H_2O_2 later in the infection process (36 and 48 h post infection with the pathogen) without any discernable differences at the earlier time points. It is reasonable to envisage that in the case of *S. sclerotiorum* infection, additional virulence factors are also involved in the elicitation of H_2O_2 production by plants, as would be expected when compared with OA alone. It is also possible that the concentration of OA secreted by the pathogen is well below that used in these studies, and/or other factors secreted by the pathogen could negate the ability of OA to inhibit H_2O_2 production. Nevertheless, OA by itself is capable of inhibiting H_2O_2 production by the host, at least with the concentrations tested in this study.

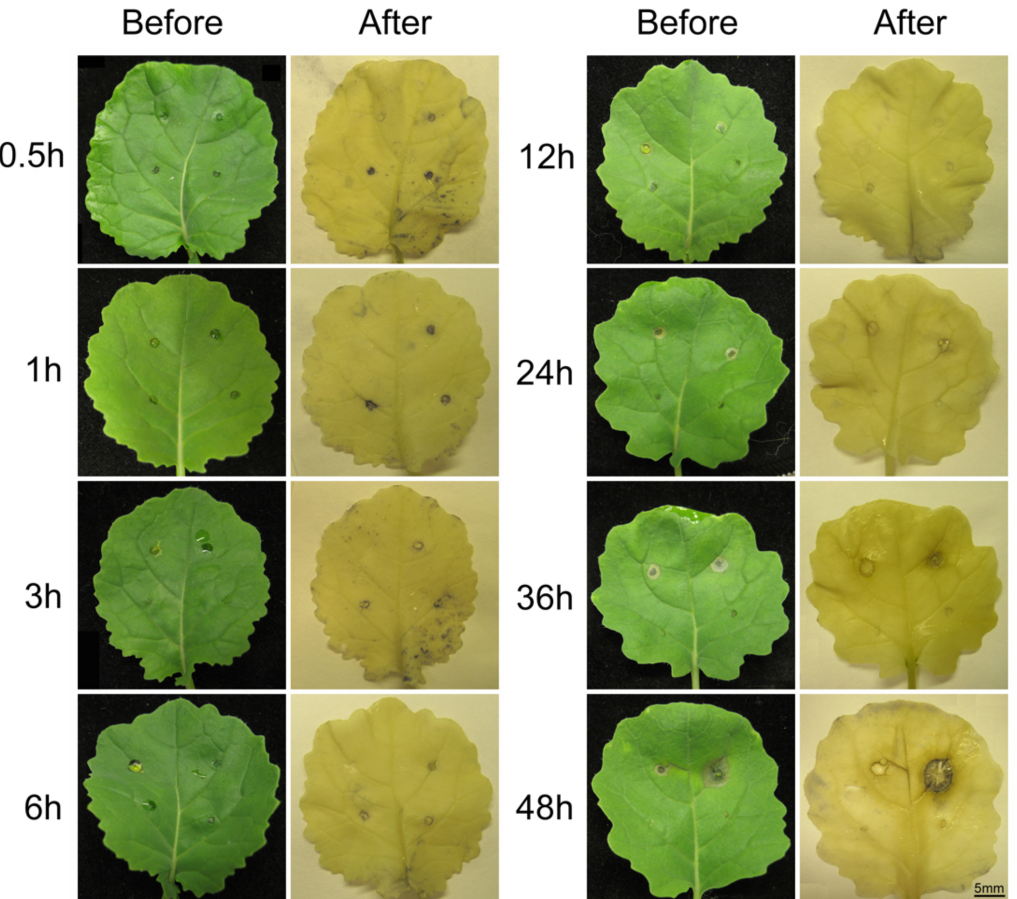

Figure 3-4. Accumulation of oxidants in leaves of *B. napus* in response to OA and *S. sclerotiorum*. A droplet (5 µL) of OA and an *S. sclerotiorum* plug were placed on either side of the primary leaf vein for the specific durations indicated. OA treatment was on the left side (top) with water control (bottom) whereas the *S. sclerotiorum* inoculation was on the right side (top) with PDA control (bottom). Representative photographs were taken before and after removing endogenous chlorophyll to increase the contrast. The accumulation of oxidant in the leaf tissue is indicated by the presence of insoluble oxidized dark blue NBT deposits.

Enzymes such as oxalic acid oxidase (OXO) and those related to ROS production and scavenging, including CAT, SOD, POX, and NADPH oxidase (NOX), are part of an active strategy used by plants to produce and remove ROS during the early phases of infection. In order to evaluate the effects of OA on the dynamics of ROS production and removal, we assayed the activities of some of these enzymes or measured their transcript abundance by real-time PCR in leaves treated with OA or inoculated with *S. sclerotiorum*. OA did not significantly affect the activity of CAT, SOD and POX at any of the time-points examined, except for a slight elevation in POX activity at 48 h; however, the relative change in POX activity at this time point was still below 1.2-fold (Fig. 3-5A). These results correlate well with the proteome results with none of the aforementioned proteins identified among the list of proteins (Table 3-2). The changes in OXO and NOX transcript abundance in response to OA and *S. sclerotiorum* were investigated using real-time PCR (Fig. 3-5B). However, neither OA treatment nor pathogen challenge had any statistically significant ($p < 0.05$) effect on the abundance of OXO transcript. The abundance of NOX transcript was significantly affected at 6 h after OA treatment, as evidenced by a sharp increase at this time point followed by a decrease to the baseline expression level. The greatest change in NOX transcript level in response to inoculation with *S. sclerotiorum* was observed at 24 h.

3.3.4. Transcriptional Analysis of Signaling Responses

Plant exposure to biotic or abiotic stresses has been demonstrated to trigger signaling cascades mediated by hormones such as salicylic acid (SA), jasmonic

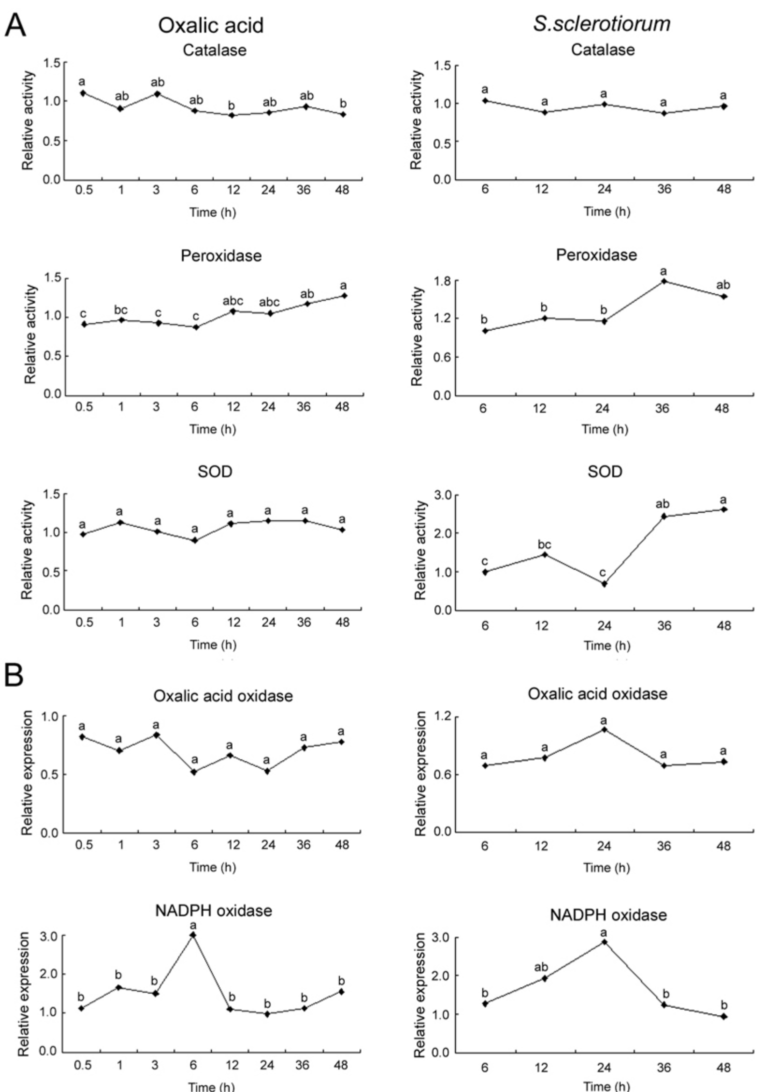

Figure 3-5. Evaluation of OA- and *S. sclerotiorum*-mediated changes in activities and/or transcript levels of *B. napus* enzymes involved in oxidative responses. Activities (A) of CAT, SOD, POX and changes in abundance of transcripts (B) of OXO and NOX are presented. Significant differences as determined by ANOVA ($p < 0.05$) observed at various time points during the entire course of the experiment are indicated by the different letters. Data used for relative changes in activity of *B. napus* POX and SOD in response to *S. sclerotiorum* were generated in an earlier study (Liang et al. 2008).

acid (JA), ET and ABA. In order to characterize the specific hormone-mediated signaling that accompanies OA treatment, we performed real-time PCR to monitor changes in transcript abundance for genes known to be related to different hormone-responsive signaling pathways (Fig. 3-6). These included *NPR1*, *TGA5*, and *TGA6* for SA signaling (Cao et al., 1994; Johnson et al., 2003; Zhang et al., 2003), *PDF1.2* and *AOC* for JA signaling (Schaller, 2001), *EIN2*, *ERF2* and *ERF4* for ET signaling (Alonso et al., 1999; Fujimoto et al., 2000), and *ABI5* for ABA signaling (Finkelstein and Lynch, 2000). Changes in the relative expression of all three genes (*NPR1*, *TGA5*, and *TGA6*) known to respond to SA were not significantly ($p < 0.05$) affected at any of the time points tested. These results, with respect to SA response, correlated well with the proteome results in that the aforementioned three proteins were not among the list of proteins identified as showing significant changes on 2-DE gels (Table 3-2). However, no significant change in *PDF1.2* transcript abundance was detected prior to 36 and 48 h, when a significant increase was observed. The *AOC* transcript abundance also increased at most time points except for 0.5, 36 and 48 h, peaking at 6 h after OA treatment. *ERF2* and *ERF4* transcript abundance increased between 6 and 12 h, whereas *EIN2* showed no significant changes at any time point. The abundance of the *ABI5* transcript gradually increased throughout the time course, with the highest levels observed at the later time points.

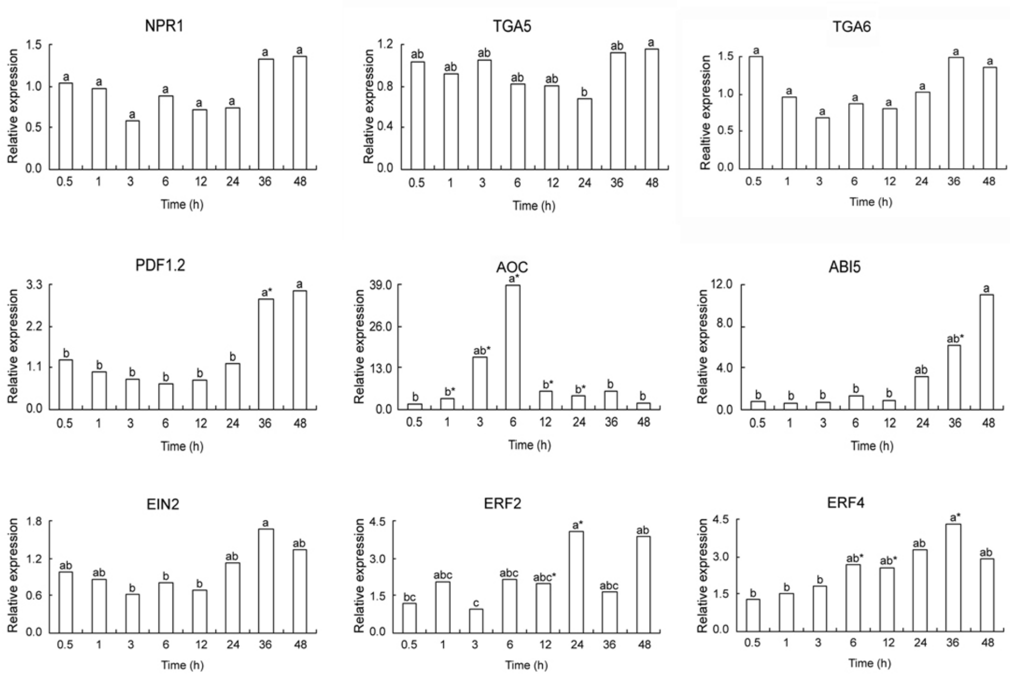

Figure 3-6. Changes in abundance of signaling gene transcripts in response to OA treatment. Relative abundance of transcripts for each gene is shown and those indicated by an asterisk (*) showed a significant increase in relative abundance (i.e., more than 1.5-fold) at a specific time point ($p < 0.05$), as determined by the Student's *t*-test. Significant differences as determined by ANOVA ($p < 0.05$) observed at various time points during the entire course of the experiment are indicated by the different letters.

3.4. Discussion

Proteomics-based studies involving 2-DE analysis combined with mass spectrometry have great utility for investigating proteins that modulate a biological response. Many examples exist in the literature that demonstrate the viability of proteome-level studies in uncovering novel information regarding cellular processes occurring in plant responses to various stresses (Liang et al., 2008; Tatlor et al., 2005; Yan et al., 2006). To our knowledge, this is the first proteome-level analysis of the host response to OA. Treatment with OA induced significant changes in the relative abundance of a number of *B. napus* proteins involved in a wide variety of processes, including protein processing, RNA processing, photosynthesis, signal transduction, the stress response and redox homeostasis.

A previous proteomic analysis of the *B. napus*/*S. sclerotiorum* interaction revealed that a number of the differentially abundant proteins between inoculated and noninoculated tissue were involved in photosynthesis (Liang et al., 2008). Some of these proteins were degraded by OA stress in the present study. The degradation of a few key proteins might be accelerated in response to external stress, possibly resulting in decreasing photosynthetic efficiency, thereby reducing the accumulation of ROS (Sweetlove et al., 2002). Therefore, OA might reduce the expression levels of proteins involved in photosynthesis, which would likely affect host photosynthetic capacity.

A molecular chaperone (HSP 70) has been indicated to be responsive to stress (Vierling, 1991). HSP70 identified in this study might be modulated by OA

stress or by the accumulation of pathogen secretions during infection. However, it was not identified as being differentially expressed in a *B. napus*/*S. sclerotiorum* proteome analysis (Liang et al., 2008), although HSP70 was observed to have increased abundance in a transcript level study of *B. napus*/*S. sclerotiorum* interaction (Yang et al., 2007).

Additionally, we identified some proteins included in signaling transduction. However, the hormone-responsive genes identified in transcriptomics studies as being differentially expressed in the *B. napus*/*S. sclerotiorum* interaction (Yang et al., 2007; Zhao et al., 2007) were not found in this proteome-level study. The results of transcript abundance suggest that SA does not appear to play a significant role in the response to OA stress, but modulation of JA, ET, and ABA could be observed in response to OA application with JA-responsive transcripts exhibiting the largest observed degree of changes. These results are consistent with the previously described proteome (Liang et al., 2008) and transcriptome (Yang et al., 2007) studies, which also indicated that SA-mediated pathways do not appear to respond in this pathosystem.

To further elucidate the effects of OA on the transcript abundance for key genes involved in phytohormone-mediated signaling pathways, we performed real-time PCR. It has previously been demonstrated that a variety of plant defense responses are regulated by the signaling molecules belonging to two major pathways: (i) the SA-dependent pathway and (ii) the SA-independent pathway involving JA and ET (Kunkel and Brooks, 2002). SA has long been known to play a critical role in plant defense, as evident by induction of local

defense and establishment of systemic acquired resistance and the expression of pathogenesis-related genes dependent on SA (Kunkel and Brooks, 2002).

The lack of changes in the transcript abundance of marker genes involved in SA signaling observed in this study is consistent with the previous proteome-level study of inoculated host tissues, which failed to identify any SA-responsive proteins (Liang et al., 2008). These results are, as mentioned previously, also consistent with the current proteome-level observations. Similarly, no SA-responsive genes were reported from gene expression profiling of the response of *B. napus* to *S. sclerotiorum* (Yang et al., 2007; Zhao et al., 2007). It has previously been predicted that SA-dependent responses do not contribute to the observed resistance to necrotrophic pathogens, such as *B. cinerea*, which is another OA-secreting pathogen (Glazebrook, 2005). Furthermore, the activation of SA-dependent plant defense depends on the specific pathosystem, which suggests that what occurs in one pathosystem might not be applicable to another, even a closely related system (Achuo et al., 2004).

SA and JA along with ET have synergistic and antagonistic interactions during signaling, which leads to a fine-tuning of the plant defense response (Kunkel and Brooks, 2002). These results along with other studies suggest that JA plays a role in plant response to OA and necrotrophic fungal pathogens (Liang et al., 2008; Yang et al., 2007; Zhao et al., 2007). JA is known to play important roles in plant development and in response to biotic stress (Creelman and Mullet, 1997). ET functions in a diverse range of plant physiological processes, including the defense response to internal or external stimuli (Broekaert et al., 2006). ET

has been demonstrated to inhibit stomatal closure and also reduces photosynthetic activity in some plants (Pallas and Kays, 1982). We observed that OA treatment results in increased levels of ET-responsive transcripts at early time-points following OA stress, which might contribute to the inhibition of stomatal closure caused by OA-secreting fungi such as *S. sclerotiorum* (Guimarães and Stotz, 2004). Interestingly, a plant defensin gene (*PDF1.2*) was reported to be activated by the concomitant triggering of the JA and ET pathways in *Arabidopsis* (Penninckx et al., 1998), and was demonstrated to play a role in the later stages of the plant response to OA stress.

ABA plays an important regulatory role, such as in the control of stomatal opening and the initiation of adaptive responses to various stresses (Mauch-Man and Mauch, 2005). Increased amounts of ABA transcript have been suggested to cause increased susceptibility to *B. cinerea*, and to negatively modulate SA-dependent defense signaling in tomato upon *B. cinerea* infection (Audenaert et al., 2002). Genetic analyses have also demonstrated that ABA negatively regulates JA-responsive defense genes and has an antagonistic interaction with ET in *Arabidopsis* (Anderson et al., 2004). The results show that an ABA-responsive gene might negatively regulate SA-responsive genes in susceptible plants, but does not appear to antagonistically modulate JA- and ET-responsive genes. The observed effect on ET-responsive transcripts, which differs from the effect seen in *Arabidopsis,* might reflect the complexity of signaling networks and/or pathosystem-specific differences. The discussion on the transcript level changes of phytohormone (JA, ABA, and ET)-responsive genes is based on the

assumption that the observed changes were the results of OA treatment alone. However, it is possible that OA-induced necrosis might also influence the abundance of transcripts for these genes, especially at later time points. Additionally, two drought-induced proteins were identified in the current proteome analysis, consistent with the suggestion that OA might alter guard cell activity to induce stomatal opening leading to water deficiency (Guimarães and Stotz, 2004).

The oxidative burst is generally considered to be one of the early responses of plants to biotic and abiotic stress, and produces a rapid, transient, and significant amount of ROS (Wojtaszek, 1997). ROS can be affected by various enzymes including NOX and SOD, and can be scavenged by other enzymes such as POX and CAT (Møller et al., 2007). Additionally, OXO can produce H_2O_2 and can be construed as a marker of the general defense response, although it is not related to the oxidative burst (Wojtaszek, 1997). In the current study, the activity of proteins involved in ROS activity did not appear to be affected by treatment with OA. However, a previous study indicated that POX protein abundance and activity as well as SOD activity increased during *S. sclerotiorum* infection in *B. napus* leaves (Liang et al., 2008). Although a previous study demonstrated that OA suppresses the oxidative burst in plants, the identities of the specific proteins involved in the observed suppression were not determined (Cessna et al., 2000). This study has expanded upon the previous study by clearly identifying which enzymes are likely contributing to the suppression of oxidative burst. Suppression of the oxidative burst by OA may also help to explain the wide host

range of *S. sclerotiorum* and other OA-secreting pathogens. Other pathogen-produced factors might contribute to the accumulation and increase in activity of POX and SOD at later stages of the infection process by *S. sclerotiorum* (Liang et al., 2008), but are absent when host tissues are treated with OA by itself. Additionally, the up-regulation of NOX and ABA-responsive transcripts (Fig. 3-5 and 3-6) observed in the *B. napus* response to OA may have resulted in increased levels of H_2O_2, which is known to mediate ABA-induced stomatal closure (Pei et al., 2000). The roles of OA secreted by *S. sclerotiorum* or other OA-secreting pathogens may change during infection. OA may initially inhibit the oxidative burst to promote pathogen growth in the host, and later induce PCD of the plant cells, facilitating tissue colonization (Kim et al., 2008; Glazebrook, 2005). Moreover, although the effects of the ROS may serve to efficiently halt further infection by biotrophic pathogens, they may not inhibit, and may even promote, infection by necrotrophic pathogens (Broekaert et al., 2006).

3.5. Concluding remarks

A model showing the possible molecular events occurring under OA stress based on the results of this study is presented in Figure 3-7. The interactions indicated in this figure may influence various cellular events. The current proteome-level investigation of *B. napus* under OA stress demonstrates that OA can affect oxidative responses, which is indicative of plant defense responses. The observed suppression of selected proteins involved in oxidative responses (SOD, OXO, CAT, and POX) and the induction of others (NOX) would affect the

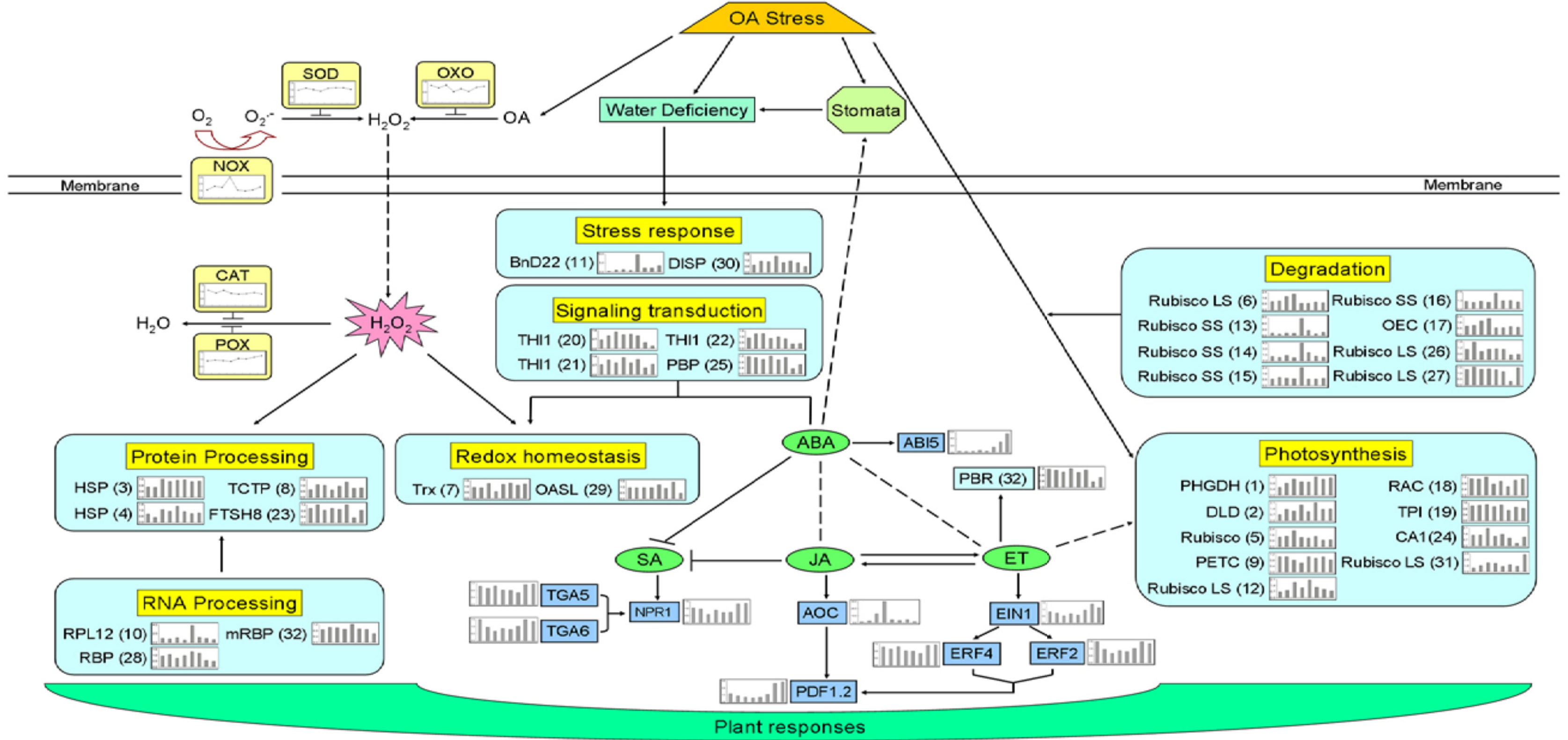

Figure 3-7. A model for the proposed events in *B. napus* leaves in response to treatment with OA. Identified proteins are indicated in boxes, the graph indicates the fold-change in abundance at each time point and numbers refer to the specific proteins whose identities are presented in Table 3-2.

accumulation of H_2O_2. Any changes to H_2O_2 abundance may have consequences on redox homeostasis and protein processing, in which RNA processing may also be involved. OA may also cause a water deficiency, based on its known induction of stomatal opening; it is interesting that among the proteins identified in this study were drought-responsive proteins. Additionally, the results appear to indicate that photosynthetic proteins are significantly altered by OA stress, with some of them potentially degraded. The precise effects of OA stress on signaling responses are not completely understood, likely as a consequence of the complexity of the signaling network. However, the results demonstrate that phytohormones (SA, JA, ET, and ABA) and proteins related to signal transduction are indeed mediated by OA stress. Future studies of the various components identified in this study may provide additional and novel insights into plant stress responses, which may in turn contribute to the development of integrated resistance to plant pathogens.

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4. Disruption of a gene encoding a hypothetical secreted protein from *Sclerotinia sclerotiorum* reduces its virulence on *Brassica napus* canola

4.1. Introdution

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungal phytopathogen that can infect more than 400 different plant species (Boland and Hall, 1994) including canola (*Brassica napus* L.), a major oilseed crop on the Canadian prairies. Sclerotia are long-term survival structures produced by *S. sclerotiorum* and serve as the primary source of inoculum during seasonal crop infection cycles (Bolton et al., 2006). Germination of sclerotia may be myceliogenic (producing vegetative hyphae) or carpogenic (producing apothecia), with the type of germination influenced by environmental conditions and crop canopy density during disease initiation (Bardin and Huang, 2001). Hyphae can directly infect plant tissue during myceliogenic germination (Huang, 1985). In carpogenic germination, millions of airborne ascospores are released from the apothecia, and in canola crops these spores land and germinate on senescing floral tissues, which serve as a nutrient source. The infected senescent tissues fall onto the leaf axils, and the pathogen mycelium grows into the stem, hindering water and nutrient uptake and often resulting in significant yield losses (Turkington and Morrall, 1993). Given its economic importance and wide host range, *S. sclerotiorum* is considered to be a model for fungal development in soilborne necrotrophs, particularly among the Ascomycetes (Broad Institute, Cambridge, MA).

The entire genome sequence of *S. sclerotiorum* has been recently annotated (Broad Institute), providing an enormous amount of data that may be used to identify putative (secreted) proteins. A number of studies have examined gene/protein expression in *S. sclerotiorum*, leading to the identification of many proteins potentially involved in virulence or pathogenicity (Li et al., 2004; Sexton et al., 2006; Yajima and Kav, 2006). Comprehensive studies of novel proteins likely require a concerted effort to integrate genomic, transcriptomic, proteomic, and metabolomic components (de Wit et al., 2009). Comparative analysis of secretomes and similarity searches of sequences can also be used to identify putative proteins or genes in sequenced genomes, although sufficient homology among genes is required for this approach to be successful (Friesen et al., 2006). Even though genome-wide searches for the identification of candidate genes or proteins involved in a particular activity are of considerable value (de Wit et al., 2009), the functions and characteristics of these putative genes and proteins still need to be characterized experimentally, using techniques that include overexpression, gene disruption or silencing, and subsequent assessment of pathogenicity on plants.

In a recent proteome-level analysis of the *Botrytis cinerea* (de Bary) Whetzel secretome (Shah et al., 2009), a number of hypothetical proteins were identified, one of which was a secreted protein (BC1G_00896.1) with a specific ortholog (SS1G_00263.1) found only in *S. sclerotiorum*. As these fungi are closely related necrotrophs with similarities in pathogenesis and virulence (Bolton et al., 2006; Williamson et al., 2007), we hypothesized that SS1G_00263.1 has a role in the

infection process and selected it for further characterization in *S. sclerotiorum*. This protein, which will hereafter be referred to as Sssp, was predicted to occur in the secretome of *S. sclerotiorum* based on the available genome database, although it has not been identified in previous studies, likely due to its small molecular mass (theoretical value of 16.5 kDa) and limitations in the methodologies used to analyze the secretome (Yajima and Kav, 2006). Furthermore, beyond the prediction that it is a secreted protein, Sssp has not been characterized in terms of activity. In this study, we describe the generation of Sssp-disrupted mutants of *S. sclerotiorum* and discuss the potential activities of this protein during the infection of a susceptible canola genotype. The mutant strains caused significantly less disease than the wild-type, suggesting that Sssp contributes to the virulence of this pathogen. To the best of our knowledge, this is the first study in which a secreted protein with unknown function has been identified as a virulence factor for *S. sclerotiorum* during the infection process.

4.2. Materials and methods

4.2.1. Fungus and growth conditions

Wild-type *S. sclerotiorum* was isolated from an infected canola stem and cultured on potato dextrose agar (PDA; Becton Dickinson, Franklin Lakes, NJ, U.S.A) for production of sclerotia (Yajima et al., 2009). The sclerotia were sliced in half and subcultured on fresh PDA for mycelial growth. To produce mycelium for DNA exaction, five mycelial plugs (5 mm in diameter) excised from the periphery of an actively growing culture of *S. sclerotiorum* on PDA were used to

inoculate 100 ml of minimal salts medium [1% wt/vol pectin (Acros Organics, Gell, Belgium), 7.3 mM KH_2PO_4, 6.7 mM KCl, 4.2 mM $MgSO_4$, 50 mM NH_4Cl, and 0.07 mM $FeSO_4$] in a 250 ml Erlenmeyer flask, which was then incubated at room temperature with agitation (100 rpm). After 5 days incubation, the cultures were harvested by filtering through Whatman #1 filter paper (Waterman International Ltd., Maidstone, U.K.). The mycelia were washed three times with sterile water by centrifugation (10,000 × *g*, 10 min, 4 °C), flash frozen in liquid nitrogen, and lyophilised overnight. The *S. sclerotiorum* disrupted-gene mutants were cultured on PDA containing 100 µg/ml hygromycin.

4.2.2. DNA extraction

Total genomic DNA from mycelium of *S. sclerotiorum* was isolated essentially as described in Rollins (2003) and Yajima et al. (2009). Lyophilized mycelium (30 mg) was placed in a sterile 1.5-ml microcentrifuge tube and ground to a powder with a sterilized metal spatula. The ground mycelium was mixed with 500 µl buffer [50 mM EDTA, 0.2% (wt/vol); sodium dodecyl sulfate, pH 8.5] and briefly vortexed. The mixture was incubated at 68 °C for 10 min and vortexed again prior to centrifugation (21,000 × *g*, 5 min, 22 °C). The supernatant was transferred to a new sterile 1.5-ml microcentrifuge tube, and a 30 µl volume of 8 M KAc, pH 10.0 was added. The mixture was placed on ice for 5 min and centrifuged as above. The supernatant was collected in a new 1.5-ml microcentrifuge tube and centrifuged again. The supernatant was carefully transferred to a new sterile 1.5-ml microcentrifuge tube and mixed with 600 µl of isopropanol. The solution was mixed vigorously and centrifuged (21,000 × *g*, 5

min, 22 °C). The pelleted DNA was air-dried and resuspended in 200 µl of Tris-EDTA buffer (100 mM Tris, 1 mM EDTA, pH 8.0) with RNase A (20 µg/ml) (Ambion, Austin, TX, U.S.A). The solution was thoroughly mixed with 10 µl of 10 M LiCl and 500 µl of 95% (vol/vol) ethanol, incubated at 37 °C for 30 min, and then centrifuged (21,000 × *g*, 5 min, 22 °C). After discarding the supernatant, the resulting pellet was air-dried and resuspended in 200 µl sterile water prior to extraction with 200 µl of phenol/chloroform/isoamyl alcohol (vol/vol; 25:24:1). Another 10 µl of 10 M LiCl and 500 µl of 95% (vol/vol) EtOH were added to the solution and it was centrifuged as above. The supernatant was discarded and the DNA pellet air-dried and resuspended in 30 µl of sterile water. The DNA concentration was measured with a NanoDrop 1000 (Thermo Scientific, Wilmingon, DE, U.S.A.) after analysis by 1% (vol/vol) agarose gel electrophoresis. DNA extraction from mature sclerotia and plant tissue collected 24 hours after inoculation with the fungus or PDA plugs (mock-inoculated controls) was performed using the same protocol.

4.2.3. Construction of the gene disruption vector

Genomic PCR was performed to amplify the *Sssp* gene, yielding a 2,315 bp fragment that commenced 889 bp upstream of the start codon and ended 1,423 bp downstream of the stop codon. The amplified fragment consisted of a 531 bp coding sequence and included the one intron present in the gene. The primers (forward, 5′-AGACAA*GCTAGC*TTTTCTTCAAGATTTATTACG-3′ with the *Nhe*I restriction site in italics; reverse, 5′-TAATAA*GAGCTC*AACGTTTCATAAATTTGATTCG-3′ with the *Sac*I

restriction site in italics) used to amplify the entire target gene from the genomic DNA were manually designed, with their specificity verified by a BLAST search. The PCR reaction was conducted using an Expand High Fidelity PCR System (Roche, Laval, Canada), with an initial heat denaturation step of 95 °C for 5 min; followed by 40 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 2.5 min; and a final extension of 72 °C for 7 min. The amplified products were visualized on a 1% (wt/vol) agarose gel and purified with a QIAquick gel extraction kit (Qiagen, Mississauga, Canada).

The purified amplicons were digested with *Nhe*I and *Sac*I (New England Biolabs, Pickering, Canada) at 37 °C for 2 h and the digests purified with a QIAquick gel extraction kit (Qiagen). The digested products were introduced into a *Nhe*I/*Sac*I-digested pET28a vector (Novagen, Mississauga, Canada) using the Rapid DNA Ligation Kit (Roche) according to the manufacturer's instructions. Competent DH5α *Escherichia coli* cells (50 µl) were transformed with 10 µl of the ligation mix and plated on Luria-Bertani agar containing kanamycin (50 µg/ml) (Invitrogen, Burlington, Canada) for overnight growth at 37 °C. Bacterial colonies were randomly selected for use in colony PCR using the same conditions as above in order to confirm that they contained the desired *Sssp*-pET28a construct. Plasmid DNA from PCR-verified colonies were isolated using the Qiaprep Spin Miniprep Kit (Qiagen) and sequenced using a BigDye Terminator V3.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, U.S.A.) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

The *Sssp*-pET28a construct and the hygromycin phosphotransferase (*hph*)-containing pCB1003 vector (Carroll et al. 1994; Fungal Genetics Stocks Center, University of Missouri, Kansas City, U.S.A.) were digested with *Kpn*I and *Sal*I (New England Biolabs), respectively, at 37 °C for 2 h and purified with a QIAquick gel extraction kit (Qiagen). The digested products were treated with the Klenow fragment of DNA polymerase I (New England Biolabs) at 25 °C for 15 min to produce the fragments required for successful blunt end ligation, and were then purified as above. A 3 μl aliquot of each sample was visualized on a 1% (wt/vol) agarose gel, while the remainder was used in blunt end ligation with a Rapid DNA Ligation Kit (Roche). Transformation of competent DH5α *E. coli* cells and colony PCR were performed as described above. Amplicons obtained from colony PCR were expected to be approximately 3,700 bp in size (2,315 bp *Sssp* fragment and ~1,400 bp *hph*), and the plasmids purified from colonies in which a PCR product was obtained were sequenced to verify the insertion of *hph* into the *Sssp* fragment.

4.2.4. Fungal protoplast preparation

Protoplasts of *S. sclerotiorum* were prepared as described in Rollins (2003) and Yajima et al. (2009). Briefly, five plugs (4 mm in diameter) excised from the periphery of a fungal colony growing actively on PDA medium were used to inoculate 25 ml of potato dextrose broth (PDB; Becton Dickinson) in a sterile Petri dish, which was incubated without agitation at room temperature. After four days, the mycelial mat growing on the surface of the medium was collected by gently peeling it off with sterile forceps. The mycelium was placed on four layers

of sterile cheesecloth on a funnel and washed with sterile water followed by protoplast buffer (0.8 M $MgSO_4 \cdot 7H_2O$, 0.2 M $Na_3C_6H_5O_7 \cdot 2H_2O$, pH 5.5).

The washed mycelium was cut into pieces with a sterile scalpel blade, and the smaller fragments were transferred to a sterile 125-ml Erlenmeyer flask. Protoplasting solution [17 ml of protoplast buffer and 3 ml buffer (1 M sorbitol, 50 mM sodium citrate, pH 5.8) containing 200 mg lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich, St. Louis, MO)] was added to the flask, which was incubated at 28 ^{o}C with gentle agitation (100 rpm) for 3 hours. Protoplasts were collected in a sterile 125 ml Erlenmeyer flask by filtering the solution through sterile cheesecloth to remove any residual hyphae. Subsequently, 30 ml of 0.6 M KCl was poured over the cheesecloth and collected in the protoplast-containing flask. The protoplast solution was transferred to 50 ml plastic tubes and centrifuged at 3000 ×*g* for 10 min at 4°C. The resulting protoplast pellet was rinsed twice with 10 ml STC buffer (50 mM $CaCl_2 \cdot 2H_2O$, 1 M sorbitol, 50 mM Tris, pH 8.0) and re-suspended in 10 ml STC buffer. The protoplast concentration was determined using a Bright Line Hemocytometer with a 0.4 mm cover slip (Fisher Scientific, Ottawa, Canada), and adjusted to a final concentration of 1×10^8 protoplasts/ml with STC buffer. For each 1 ml of protoplast solution, 12.5 µl dimethyl sulfoxide (DMSO), 62.5 µL heparin (5 mg/ml in STC buffer), and 250 µl polyethylene glycol (PEG) solution (40% wt/vol PEG 4000, 0.6 M KCl, 50 mM Tris, pH 8, 50 mM $CaCl_2$) were added, and the protoplasts were stored at –80 ^{o}C until needed.

4.2.5. Fungal protoplast transformation

The *Sssp-hph*-pET28a construct was double-digested with *Nhe*I and *Sac*I and the linearized *Sssp-hph* fragment purified with a QIAquick gel extraction kit (Qiagen). Fungal protoplast transformation was performed as described in Rollins (2003) and Yajima et al. (2009). Briefly, 100 μl of a 1×10^8 protoplast/ml suspension in STC buffer was mixed with 5 μg of the linearized *Sssp-hph* fragment and incubated on ice for 1 h. PEG (1 ml) was gently mixed into the solution, which was then incubated at room temperature for 20 min. The suspension was spread evenly onto the surface of 25 ml regenerating medium (RM) [0.7 M sucrose, 0.05% (wt/vol) yeast extract, 1.5% (wt/vol) agar] bottom agar in Petri dishes and then incubated overnight in darkness at room temperature. Five ml of RM top agar [0.7 M sucrose, 0.05% (wt/vol) yeast extract, 0.8% (wt/vol) agar and 0.6 mg/ml hygromycin B] was spread evenly over the entire surface of each Petri dish, which was then incubated at room temperature until colonies were observed. The hyphal-tips of colonies that emerged through the RM top agar were carefully excised and transferred onto PDA plates (containing 100 μg/ml hygromycin B), and subsequently regenerated four more times on PDA containing hygromycin B. A protoplast suspension mixed with buffer, but containing no linearized *Sssp-hph*, was spread and incubated on RM bottom and then top agar with or without hygromycin; these treatments served as the corresponding controls for protoplast growth and the hygromycin-treated condition.

6.2.6 Determination of *hph* integration events

To determine whether successful *hph* integration into the genome of *S. sclerotiorum* had occurred, genomic DNA from the wild-type and three representative transformants was extracted as described earlier. The *Sssp*-specific primers (forward, 5′-ATGCATTTCTCCATGATTGC-3′; reverse 5′-AAGCATAATCGGTGTAGC-3′) were used in genomic PCR to amplify part of the *Sssp* gene under the following reaction conditions: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; and 72 °C for 10 min. An amplicon about 1,800 bp in length was expected if *hph* was successfully integrated into *Sssp*, while an amplicon only 457 bp in length was expected from genomic DNA of the wild-type. The *hph*-specific primers (HPH: K01193; forward, 5′-CAAAGCATCAGCTCATCGAGAG-3′; reverse, 5′-GAAAAGTTCGACAGCGTCTCC-3′) were expected to generate a PCR product 502 bp in length only from genomic DNA of the mutant strains under reaction conditions consisting of 95 ^{o}C for 5 min; 30 cycles of 95 ^{o}C for 1 min, 54 ^{o}C for 1 min, 72 ^{o}C for 1 min; and 72 °C for 10 min. Amplification of *actin* (SS1G_08733) using primers (forward, 5′-GTTGGAGATGAAGCGCAATCCAAG-3′; reverse 5′-CTCAAGACCCAAGACAGATGGTTGG-3′) designed to generate a product 651 bp in length was conducted under conditions consisting of 95 ^{o}C for 5 min; 30 cycles of 95 ^{o}C for 1 min, 56 ^{o}C for 1 min, 72 ^{o}C for 1 min; and 72 °C for 10 min.

The effect of *hph* integration on expression of *Sssp* was evaluated by reverse transcriptase (RT)-PCR. RNA was extracted from wild-type and mutant mycelium of *S. sclerotiorum* using an RNeasy Plant Mini Kit (Qiagen), with a

DNase treatment included to remove contamination by DNA. cDNA was synthesized with the iScript cDNA Synthesis Kit (BioRad, Mississauga, Canada) according to the manufacturer's procedure. Amplification of *Sssp*, *hph* and *actin* gene was performed using the gene-specific primers and reaction conditions described above. Extracted RNA was also used as a template for PCR to confirm that there was no DNA contamination. All PCR experiments were performed using the Expand High Fidelity PCR System (Roche). The presence of *Sssp* was also confirmed in mature sclerotia and canola tissue 24 hour post inoculation (hpi) with the wild-type fungus or a water control.

4.2.7. Infection assays on host plant

Seeds of *Brassica napus* L. doubled haploid line DH12075 were planted individually in 13 cm diameter plastic pots filled with LA4 Aggregate Plus Sunshine Professional Peat-Lite Mix (Sungro Horticulture, Vancouver, Canada). The plants were maintained in a growth chamber under an 18 hour photoperiod (21 ^{o}C day/18 ^{o}C night) and fertilized with 15-30-15 (Plant Products Co., Brampton, Canada) every week. Fully expanded leaves were excised at the six-true leaf stage and placed in 150 × 15 mm Petri dishes lined with water-moistened sterile no. 1 Whatman filter paper. A sterile cotton ball was saturated with water and placed at the base of each leaf. Each excised leaf was inoculated with one agar plug (4 mm in diameter) of actively growing mycelium of the wild-type (grown on PDA) or mutant strains of *S. sclerotiorum* (grown on PDA with 100 µg/ml hygromycin). The Petri dishes were sealed with plastic laboratory film and incubated at room temperature for a 72 h period.

Leaves were photographed at 24 h intervals, and the lesion (necrotic) area was quantified using image analysis software (APS Assess: Image Analysis Software for Plant Disease Quantification; APS Press, St. Paul, MN) as per the manufacturer's instructions. Three mutant strains of the fungus were used in the inoculation assays and were regarded as independent biological replicates; a wild-type strain of *S. sclerotiorum* was also included in each assay, as was a mock-inoculated control (inoculated only with PDA). Three replicates were included in the inoculation trials, with three leaves per replicate per fungal strain. Treatments were arranged in a randomized complete block design. The lesion data were analyzed using the Student's *t*-test ($P < 0.01$) in SAS software version 9.1 (SAS Institute Inc., Cary, NC).

4.3. Results

4.3.1. Sequence analysis and gene confirmation

Hypothetical proteins with unknown functions identified in the secretome of *B. cinerea* (Shah et al., 2009) were compared to the *S. sclerotiorum* genome database. One of the proteins (SS1G_00263.1) identified by BLASTP analysis of the NCBI database was an ortholog found only in *S. sclerotiorum* and *B. cinerea* (E-value = 2×10^{-60} with an identity of 72%). This result suggests possible functional conservation of this protein in these two necrotrophic plant pathogens. The *Sssp* gene is located on Supercontig 1 (681499-682029+) with a coding sequence 531 nt in length and a 63 nt intron near the stop codon. The gene encodes a 155 aa protein with a putative signal peptide (Broad Institute), and had

no significant homology with any Genbank accessions except for the original protein identified from *B. cinerea* (BC1G_00896.1).

The presence and expression of *Sssp* in vegetative hyphae and sclerotia of wild-type *S. sclerotiorum*, as well as in host leaf tissue 24 h after inoculation with the wild-type fungus, were assessed by genomic and RT-PCR with *Sssp*-specific primers. The amplification results revealed that the *Sssp* gene and transcript were present in the wild-type fungus and infected plant tissues, but were absent in mock-inoculated (control) leaf tissue (Fig. 4-1). As there was no annotation of Sssp in the available databases beyond its gene sequence, gene-disruption studies were undertaken to determine whether this protein plays a role in the infection process.

4.3.2. Fungal transformation

Transformation of *S. sclerotiorum* protoplasts was performed with the linearized *Sssp-hph* DNA fragment for homologous recombination leading to the integration of *hph* into the genome within *Sssp*. Transformants started to emerge through the RM top agar 3 days after plating, as determined by examination under a stereomicroscope. The first colonies were visible to the naked eye 5 days after plating, with additional colonies observed on subsequent days. Regenerating colonies were tip-excised and transferred onto fresh PDA plates containing hygromycin. In order to confirm that the hygromycin concentration was appropriate, a control was included in which a mixture of DNA elution buffer (without the linearized *Sssp-hph* DNA fragment) and protoplasts of *S. sclerotiorum* was plated onto RM containing hygromycin. A

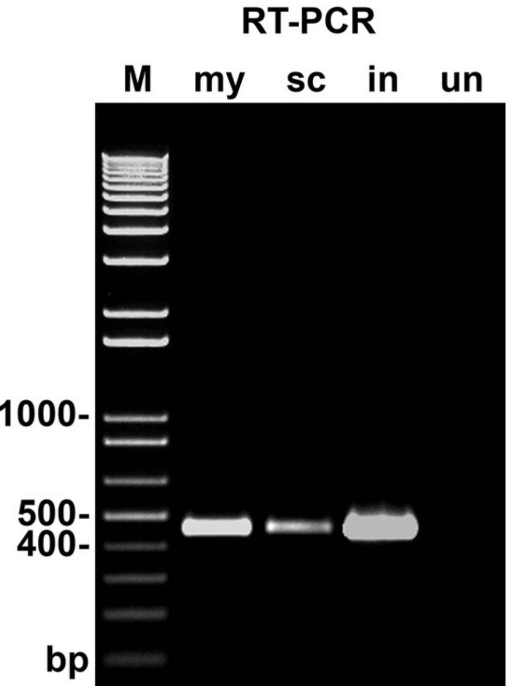

Figure 4-1. Confirmation of the production of *Sssp* by wild-type *S. sclerotiorum* via reverse transcriptase (RT)-PCR. An amplicon of the expected size (~450 bp) was obtained from wild-type mycelium (my), sclerotia (sc) and canola (*Brassica napus*) leaf tissue 24 hours post-inoculation (hpi) with the wild-type strain (in); in contrast, no amplicon was obtained from mock-inoculated leaf tissue at 24 hpi (un) or any other time. The size marker (M) included was a 1-kb Plus DNA ladder (Invitrogen).

second control was also included in which the mixture of DNA elution buffer (without the linearized *Sssp-hph* DNA fragment) and protoplasts was plated onto RM lacking hygromycin. No regenerating colonies were observed emerging through the RM top agar in the first control treatment, while mycelial colonies were observed in the second control 3 days after plating; thus, the concentration of hygromycin was sufficient to prevent non-transformed fungal protoplasts from growing, and the protoplasts were viable.

4.3.3. Genomic and RT-PCR

The wild-type and mutant strains were analysed by genomic and RT-PCR. The genomic PCR results are shown in Figure 4-2A, where a single amplification product (*Sssp-hph* hybrid; about 1,850 bp in length) was observed from the mutant using *Sssp*-specific primers, while another single amplification product (~450 bp) was generated from the wild-type only. The difference in size between the amplicons obtained from the wild-type versus the mutant corresponded to the expected size (~1,400 bp) of the integrated *hph* gene. RT-PCR revealed that the *Sssp* transcript was present only in the wild-type. A single and faint amplification product (1,850 bp), which was sequenced and confirmed to be an *Sssp-hph* hybrid (data not shown), was observed in the mutants.

In order to confirm the presence of *hph* in the mutants, *hph*-specific primers were used for genomic and RT-PCR and a single amplification product of the expected size (~500 bp, a partial fragment of the entire *hph*) was observed for the mutants only (Fig. 4-2B). These results revealed that *hph* was successfully integrated into the genome and that the *hph* transcript was expressed only in the

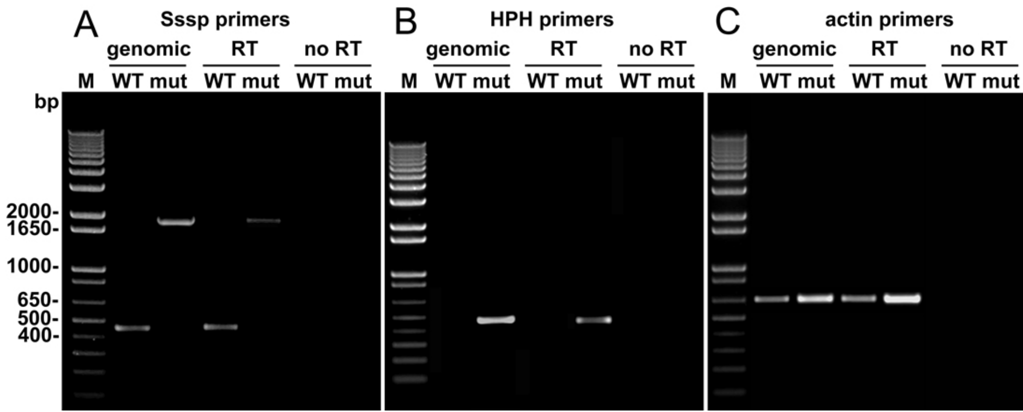

Figure 4-2. PCR amplification assays. A, When using *Sssp*-specific primers, an amplification product of the expected size (~450 bp) was obtained from genomic and reverse transcribed (RT) cDNA of the wild-type strain (WT), while a product ~1,850 bp in size, corresponding to the *Sssp* gene with the hygromycin phosphotranferase (*hph*) insertion, was obtained from the mutant strain (mut). B, When using *hph*-specific primers, the *hph* gene fragment and transcript were amplified only from the mutant, further indicating successful insertion of *hph*. C, When using actin-specific primers as a control, a product of the expected ~650 bp size was obtained from both the wild-type and mutant strains of *Sclerotinia sclerotiorum*. The absence of a detectable amplicon in the no-RT controls indicates that the bands obtained by RT-PCR were not the result of DNA contamination. The size marker (M) included was a 1-kb Plus DNA ladder (Invitrogen). The results presented are for a representative mutant strain; identical results were obtained with two other strains (data not shown).

mutant. Moreover, given the amplification of the *actin* internal control from all treatments, the absence of *Sssp* or *hph* amplification products was not caused by poor quality or insufficient cDNA (Fig. 4-2C). As expected, no amplification products were observed when RNA was used as the template (e.g., no-RT control), which indicated that amplification products from the RT-PCR were not caused by contamination with genomic DNA (Fig. 4-2). All three potential mutants producing an amplicon of the expected size were used in subsequent leaf inoculation experiments; each mutant was considered as an independent biological replicate.

4.3.4. Infection assays on host plants

Typical water-soaked lesions were observed at all time-points on leaves inoculated with the wild-type or mutant strains, while cottony white mycelium was observed only at 72 hpi (Fig. 4-3). The lesions that developed on leaves inoculated with the mutants were significantly ($p < 0.01$) smaller than those that developed after inoculation with the wild-type at all time-points (Fig. 4-4).

4.4. Discussion

Plant pathogens utilize a wide variety of mechanical or biochemical strategies to effectively attack host plants. Necrotrophic pathogens release a number of cell wall-degrading enzymes (e.g., hydrolases and lyases) to break down the plant cell walls, which are composed primarily of an integrated matrix of polysaccharides

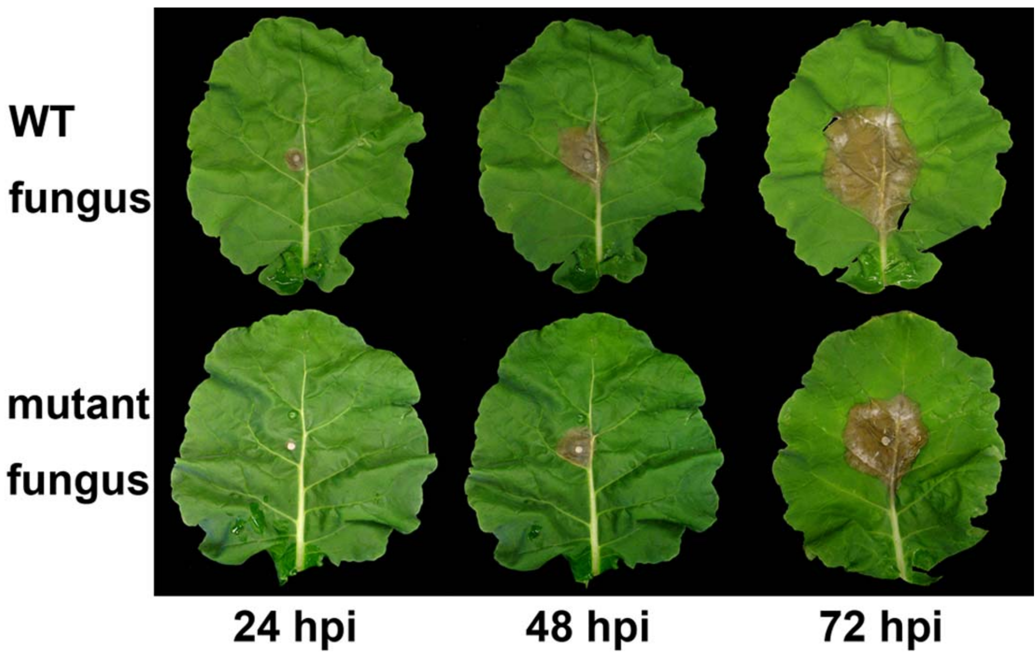

Figure 4-3. Symptom development on canola (*Brassica napus*) leaves inoculated with a wild-type (WT) or *Sssp* gene-disrupted mutant strain of *Sclerotinia sclerotiorum*. The images show the appearance of leaves at 24, 48 and 72 hours post-inoculation (hpi).

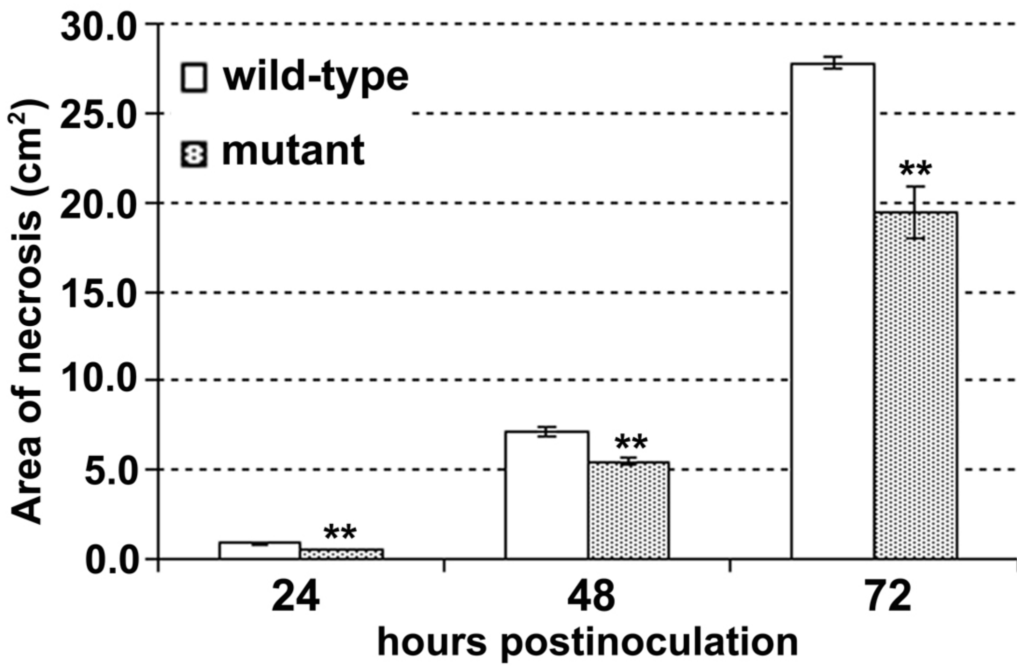

Figure 4-4. Development of necrosis on canola (*B. napus*) leaf tissue inoculated with a wild-type or *Sssp* gene-disrupted mutant strain of *S. sclerotiorum* at 24, 48 and 72 hours postinoculation. The area of necrosis was measured using Assess Image Analysis Software for Plant Disease Quantification (APS Press). Error bars indicate the standard error of the mean, while double asterisks denote a statistically significant difference ($p < 0.01$) between treatments at each time-point as assessed by the Student's *t*-test.

including cellulose, hemicelluloses, and pectin (Cosgrove, 2005; Hématy et al., 2009). Specifically, *S. sclerotiorum* secretes a number of pectinases that degrade pectin and facilitate fungal ingress (Bolton et al., 2006). Some pathogens may also possess mechanisms to evade or inactivate host defense responses, such as the oxidative burst and the production and export of anti-microbial compounds (Hématy et al., 2009).

The current results indicate that the mutant strains are still able to infect susceptible canola tissue even in the absence of Sssp, although they cause significantly reduced symptoms relative to the wild-type. The fact that the Sssp-deficient *S. sclerotiorum* strains are still pathogenic likely reflects the secretion of other cell wall-degrading enzymes and virulence factors by this fungus (Vorwerk et al., 2004). Thus, Sssp appears to serve as a virulence factor rather than a pathogenicity factor for *S. sclerotiorum*, since this protein was not essential for disease development yet contributed to symptom severity. Other recent studies have also demonstrated that the disruption of a single gene can result in reduced virulence in *B. cinerea* and *S. sclerotiorum* (Vallette-Collet et al., 2003; Yajima et al., 2009). For instance, the disruption of a secreted arabinofuranosidase/β-xylosidase precursor secreted by *S. sclerotiorum* also caused a significant reduction in lesion size (Yajima et al., 2009).

Since Sssp has no similarity to any cell-wall degrading enzymes or other proteins with a known function, it is difficult to speculate on its precise role in the infection process. It has been observed that cell wall degradation products can modulate the production of other cell wall-degrading enzymes (Hématy et al.,

2009). Thus, it is possible that production of Sssp may somehow play a part in the accumulation of such products, thereby contributing to the amount/activity of other cell wall degrading enzymes. Alternatively, the protein may have a role in reducing or evading the host defense response, which could ultimately result in diminished symptom development in response to inoculation with the mutant strains. Additional experiments to characterize gene expression, enzyme activities, histological changes and other molecular events during infection are needed to test these possibilities.

The identification of Sssp as a virulence factor for *S. sclerotiorum* may have applications in crop protection and related industries. Firstly, Sssp could be used as a specific biomarker to detect Sclerotinia diseases at the gene or protein level, given that the encoding gene appears to be fairly unique to *S. sclerotiorum* and *B. cinerea*. Secondly, the development of an effective antibody-based application targeting Sssp could have applications as a tool to improve resistance to *S. sclerotiorum* in canola and other crops, perhaps through a transgenic approach.

The identification of Sssp as a virulence factor for *S. sclerotiorum* stemmed from an earlier proteomic analysis of *B. cinerea*, further revealing the merits of proteome-based studies in increasing understanding of plant pathogens. A comprehensive proteomics-based investigation includes not only a list of proteins identified from a particular pathogen, but also takes full advantage of the integration of genomic, transcriptomic, proteomic, and metabolomic data. Characterization of the function(s) of proteins identified using such an approach

may provide novel insights into fungal virulence and pathogenicity, and may ultimately help to develop novel disease management strategies.

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5. Developmentally induced changes in the sclerotial proteome of *Sclerotinia sclerotiorum**

5.1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungal phytopathogen that causes significant crop losses throughout the world. The host range of *S. sclerotiorum* includes over 400 species, most of which are herbaceous plants, including a substantial number of economic crops and horticultural plants (Boland and Hall, 1994). Sclerotia are long-term survival and dissemination structures that can withstand adverse environmental conditions (Willetts, 1971). The sclerotia are also known to play a crucial role in the life cycle of *S. sclerotiorum*, serving as the primary inoculum source during seasonal crop infection cycles by this pathogen (Bolton et al., 2006). The survival of sclerotia is influenced by various factors, including structural and environmental factors such as moisture, aeration, and temperature (Coley-Smith and Cooke, 1971). Environmental conditions and crop canopies can lead to myceliogenic or carpogenic germination of sclerotia (Bardin and Huang, 2001). Myceliogenic germination results in the production of hyphae or mycelia that directly infect plant tissues (Huang, 1985). Carpogenic germination of sclerotia results in the formation of apothecia that release ascospores. Germination of ascospores under high moisture conditions, combined with the presence of senescent tissues that can serve as nutrient sources, leads to the infection of aboveground plant tissues (Tu, 1989; Turkington and Morrall, 1993). Both types

*A version of this chapter has been published. Liang, Y., Rahman, M.H., Strelkov, S.E., Kav, N.N.V. 2010. Fungal Biology, doi:10.1016/j.funbio.2010.05.003

of germination can result in the initiation of diseases (Morrall, 1977; Huang and Kozub, 1993).

The sclerotium is a multiple-hyphal aggregate structure and consists of three distinct layers: a pigmented rind, a thin-walled cortex, and a large central medulla (Jones, 1970; Colotelo, 1974; Kosasih and Willetts, 1975). Three distinct stages have been recognized during sclerotial development: (i) initiation, the formation of small and discrete initials though the aggregation of hyphae; (ii) development, increasing size along with the surface exudation of liquid droplets; and (iii) maturation, involving surface delineation and melanin deposition in peripheral rind cells followed by internal consolidation (Townsend and Willetts, 1954). The induction and development of sclerotia requires specific metabolic and physiological processes. The initiation of sclerotial development is affected by several endogenous and exogenous factors including nutrients, light, temperature, pH, and aeration (Chet and Henis, 1975). Development and maturation of the sclerotia involves complex morphological and biochemical processes. These include the branching, interweaving and fusion of hyphae, translocation of nutrients and water, and the exudation of liquid droplets from the sclerotial surface (Chet and Henis, 1975; Willetts and Bullock, 1992). The biochemical composition of sclerotia has been shown to consist of carbohydrates, enzymes, free amino acids, and fatty acids (Cooke, 1969; Jones, 1970; Colotelo et al., 1971). Additionally, melanin is the darkly pigmented polymer whose deposition accompanies sclerotial development and maturation, and serves a critical role in fungal persistence and longevity by affording protection against adverse

environmental conditions (e.g., temperature extremes, UV and visible irradiation, desiccation, toxic metals, and antagonistic or competitive microbes) (Bell and Wheeler, 1986). Fungal melanin is also involved in pathogenicity and/or virulence, and is an essential requirement for appressorial formation and penetration of host plants (Butler and Day, 1998).

Proteomics is a powerful tool that can be applied to increase the understanding of complex cellular processes and networks. Although there are a number of studies on the biology and physiology of sclerotial development, little is known of the proteomic changes associated with this biological process. In the current study, a global proteome analysis of sclerotia was performed to elucidate the proteome-level changes that occur during the three distinct stages of sclerotial development: initiation, development and maturation. To our knowledge, this is the first study investigating differentially regulated proteins with an aim to obtain novel information on the molecular events occurring during sclerotial development in *S. sclerotiorum*. The results presented provide new insights into sclerotial development that will form the framework for the future characterization of the roles of specific proteins in this process, including the establishment of functions for proteins with as of yet unknown roles. Such knowledge, in the long-term, may facilitate the development of novel, alternate strategies for disease management.

5.2. Materials and methods

5.2.1. Microorganism and culture conditions

Sclerotia were harvested as described in the literature with minor modifications (Rollins and Dickman, 1998). Briefly, a mature sclerotium of the wild-type *S. sclerotiorum* was sliced into two halves using a sterile scalpel and cultured at room temperature on potato dextrose agar (PDA; Becton Dickinson; Franklin Lakes, NJ, USA), with the sectioned sides touching the medium. A mycelial plug (5 mm) was excised from the periphery of the resulting colony after 4 days and subcultured on PDA. Subsequently, sclerotia were harvested at three distinct developmental stages: initiation (4 days of growth), development (5 days of growth) and maturation (8 days of growth), and stored at –80 °C. Three individual sclerotia were used to start the mycelial cultures that served as the source of the sclerotia used in the proteome analysis. Each of the original sclerotia was considered as an independent biological replicate, and therefore three replicates were included in the proteomic analysis.

5.2.2. Protein extraction

After homogenization of the sclerotia (~300 mg) in liquid nitrogen, total protein was extracted as follows: a fine powder of ground sclerotia was suspended in 1 mL of acetone containing 0.07% (w/v) dithiothreitol (DTT, Fisher Scientific, Fair Lawn, NJ, USA) and 10% (w/v) trichloroacetic acid (TCA, Fisher Scientific) and incubated at –20 °C for 1 h. The suspension was centrifuged at 18,000*g* for 15 min at 4 ºC, and the pellet washed three times with 1 mL of ice-cold acetone containing 0.07% (w/v) DTT. The pellet was dried *in vacuo* and resolubilized in 400 μL rehydration/sample buffer containing 1.0% (v/v) tributylphosphine (Bio-Rad, Hercules, CA, USA) overnight at 4 ºC. The samples were subsequently

centrifuged at 18,000*g* for 15 min at 4 ºC and the protein concentrations in the supernatant were assessed with a protein assay dye reagent (Bio-Rad) using BSA as a standard (Yajima and Kav, 2006).

5.2.3. Two-dimensional gel electrophoresis

The proteins (300 μg) were passively rehydrated overnight in 300 μL rehydration/sample buffer and applied to IPG strips (17 cm, pH 4–7, Bio-Rad). Isoelectrofocusing (IEF) was carried out using a PROTEAN IEF cell (Bio-Rad) programmed as follows: the voltage was initially held at 250 V for 15 min, then linearly increased to 10,000 V over 3 h, focused for 60,000 Vh and held at 500 V for 1 h. Each focused strip was subsequently equilibrated in 5 mL of equilibration buffer (0.37 M Tris-HCl, pH 8.8, 6 M urea, 2% SDS, and 20% glycerol) containing 130 mM DTT with gentle agitation two times (10 min each), followed by incubation in 5 mL of equilibration solution containing 135 mM iodoacetamide (IAA, Bio-Rad) another two times (10 min each). The second dimension SDS-PAGE was performed on 13% polyacrylamide gels in a PROTEAN II xi Cell (Bio-Rad) at 25 mA per gel, until the dye front reached the bottom of the gel, at which time electrophoresis was continued for an additional 15min. The gels were stained with a Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. Two technical replicates were run for each biological replication, for a total of six gels for each developmental stage.

5.2.4. Image acquisition and statistical analysis

Gel images were recorded with a GS-800 calibrated densitometer (Bio-Rad). A matchset was created from the 18 gel images (two technical replicates of three biological replicates for each of the three developmental stages) using PDQuest software version 7.3.1 (Bio-Rad). Protein spot detection and quantification were performed using the automated feature of PDQuest followed by manual verification. Matched spots whose abundance was significantly ($p < 0.01$) altered were determined between any two of the three developmental stages employing the Student's *t*-test feature of the software. To further verify and analyze the temporal expression of significant spots generated by the PDQuest software, quantification values of the matched spots were exported and statistically analyzed using SAS software (version 9.1, SAS Institute Inc., Cary, NC, USA). The relative expression abundance for each significant spot at each of the three developmental stages was normalized as an expression ratio using samples from the sclerotial initiation or maturtion stages against the development samples (i.e., expression ratio = 4 days/5 days and 8 days/5 days, whilst the expression ratio at 5 days was set to one by default). Data for each spot were subjected to the generalized linear model (GLM) procedure for analysis of variance (ANOVA) with Duncan's multiple range test ($p < 0.05$) in order to delineate expression patterns over the three different developmental stages. All protein spots that were reproducibly and significantly altered in abundance were selected for MS/MS.

5.2.5. In-gel tryptic digestion

Protein spots showing statistically significant ($p < 0.01$) changes in abundance were excised from the stained gels and placed in 1.5 mL microcentrifuge tubes for

in-gel digestion with trypsin in a laminar flowhood. The gel pieces were washed with 100 μL of HPLC grade water (Fisher Scientific) for 5 min, dehydrated with 50 μL of 100 % acetonitrile (ACN) for 10 min, destained with 50 μL of 50 mM NH_4HCO_3/50% ACN for 10 min with periodic vortexing, and dehydrated with 50 μL of 100% ACN for 10 min followed by air-drying for 10 min. Proteins in the gel pieces were reduced by incubating with 30 μL of 0.1 M NH_4HCO_3/10 mM DTT at 56 °C for 30 min, dehydrated with 50 μL of 100% ACN for 5 min, and alkylated with 30 μL of 0.1 M NH_4HCO_3/55 mM IAA in the dark for 20 min. After rinsing with 150 μL of 0.1 M NH_4HCO_3 and occasional gentle vortexing for 15 min, the samples were dehydrated with 50 μL of 100% ACN for 10 min and air-dried; this rinse/dehydration step was repeated once more. Afterwards, the dried gel pieces were rehydrated with 30 μL of 0.02 μg/μL trypsin (Promega, Madison, WI, USA) in 40 mM NH_4HCO_3/10% ACN at room temperature for 60 min, and incubated at 37 °C overnight. The digestion was stopped by the addition of 3 μL of 2% formic acid (FA) and incubation for 30 min at room temperature. The solution was transferred into a new microcentrifuge tube and the resulting peptides were extracted twice with 15 μL of 50% ACN/0.1% FA (v/v). The peptide extracts were stored at –20 °C for subsequent protein identification.

5.2.6. LC-MS/MS and protein identification

The peptide mixtures obtained through digestion were analyzed on an Agilent 1100 Series LC/MSD Trap XCT System (Agilent Technologies, Palo Alto, CA, USA) operated in the unique peptide scan auto-MS/MS mode. An autosampler was used to inject 20 μL of peptide mixture onto a concentration column (Zorbax

300SB-C18, 5 µm, 5 × 0.3 mm), followed by a second separation column (Zorbax 300S B-C18, 5 µm, 150 × 0.3 mm). The columns were activated at a flow rate of 4 µL/min and the samples analyzed using a linear HPLC gradient (buffer A, 0.1% FA in H_2O and buffer B, 0.1% FA in CAN; v/v) under the following conditions: an initial mode of 3% buffer B for 5 min, to 15% buffer B for 3 min, to 45% buffer B for 42 min, to 90% buffer B for 10 min, to 3% buffer B for 1 min; and finally to 3% buffer B for 14 min to clean the column prior to the injection of the next sample. The peptide ion fragmentation information was acquired using an MS 300–2000 m/z scan followed by an MS/MS analysis of the most intense ions. Raw data of mass spectra were converted into Mascot Generic File (*.mgf) format using the default method in the ChemStation Data Analysis module.

All of the files generated (*.mgf) were analyzed using the MS/MS ion search module of Mascot software (version 2.1; Matrix Sciences, London, UK; http://www.matrixscience.com/) and queried against the NCBI nonnedundant (NCBInr) database, using fungi as the taxonomic category with the following search criteria: one missing cleavage allowance by trypsin, fixed modification of carbamidomethyl, ±2.0 Da peptide tolerance, ±0.8 Da MS/MS tolerance, 1+, 2+, 3+ peptide charge, monoisotopic ions with no precursor, and ESI-TRAP instrument. Individual ion scores resulting in peptide mass fingerprints obtained by the Mascot search were calculated as $-10 \times \text{Log}(P)$, where P is the probability that the observed match is a random event. Individual ions scores with a threshold value >50 were considered to indicate identity or extensive homology ($p < 0.05$) as defined by the Mascot probability analysis.

5.2.7. Bioinformatics and classification

Functional groupings of identified proteins were based on InterPro (http://www.ebi.ac.uk/interpro/) and FunCat classifications (the Munich Information Center for Protein Sequences; http://www.helmholtz-muenchen.de/en/mips/) (Apweiler et al., 2001; Ruepp et al, 2004).

5.3. Results and discussion

5.3.1. Development of sclerotia

The developmental sequence of the sclerotia was similar to that reported in a previous study (Colotelo, 1974). Mycelia grew and developed into a dispersed colony, with clumps appearing among hyphae after 3 days of growth on PDA, as described earlier (Willetts and Bullock, 1992). The sclerotial initials formed from white mycelial aggregates by 4 days (Fig. 5-1A), whilst liquid droplets of exudates started to appear and coalesce on the sclerotial surface. The number of developing sclerotia continued to increase, and their pigmentation also increased rapidly as they turned from grey to black, with a simultaneous increase in the size and number of the exudate droplets by 5 days (Fig. 5-1B). The pigmented, mature sclerotia were clearly visible on the edge of plate by 8 days of growth, at which time liquid droplets were no longer present on the sclerotial surface (Fig. 5-1C).

5.3.2. Proteome changes during sclerotial development and their functional classification

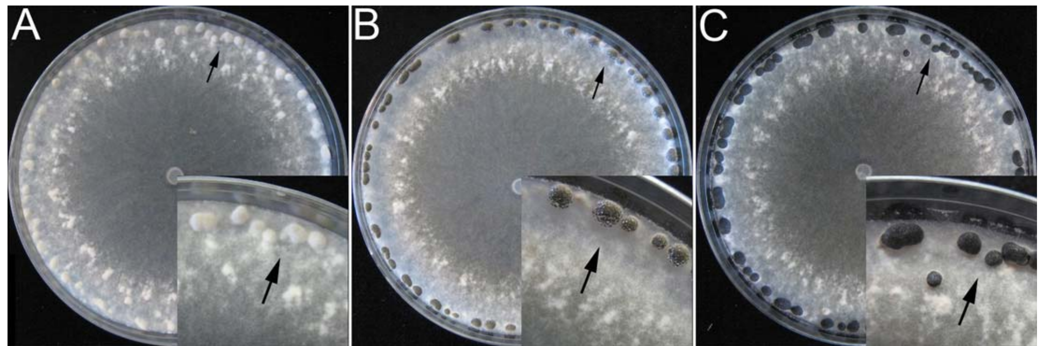

Figure 5-1. Three distinct stages in the formation of sclerotia of *S. sclerotiorum*: (A) initiation (4 days of growth); (B) development (5 days of growth); and (C) maturation (8 days of growth). The enlarged panel on the right corner shows a typical sclerotium (arrow).

Individual protein spots were resolved by 2-DE with a pH range of 4–7, with good reproducibility and resolution achieved on Colloidal blue stained gels. A representative image of a 2-DE gel is shown in Figure 5-2. PDQuest analysis revealed that a total of 719 matched protein spots could be detected on the gels over the three different developmental stages examined. Of the spots observed, a total of 88 exhibited significant differences ($p < 0.01$) in abundance between any two stages and could be identified by LC-MS/MS (Table 5-1 and Table S5-1). The results indicate that 20 of these protein spots were common to all sclerotial developmental stages investigated in this study, with 39 differing in abundance between the initiation (4 days) and development stages (5 days), 26 differing in abundance between the development and maturation stages (8 days), and 67 differing in abundance between the initiation and maturation stages (Fig. 5-3). To understand the predicted functions of the identified proteins, the differentially expressed proteins were sorted into several functional categories (Fig. 5-4).

5.3.3. Proteins associated with development

Most of the proteins identified as being involved in amino acid metabolism exhibited a significant ($p < 0.01$) decrease in abundance during the various stages of sclerotial development. Two proteins matching aminotransferase and aldehyde dehydrogenase (spots 75 and 84) showed consistent decreases in abundance at all developmental stages (Table 5-1). Saccharopine dehydrogenase (spots 80 and 82) is known to catalyze lysine biosynthesis in fungi and the deletion of the lysine biosynthesis gene (*lysF*) lead to attenuation of virulence in *Aspergillus fumigatus* (Zabriskie and Jackson, 2000; Liebmann et al., 2004). The loss of virulence in a

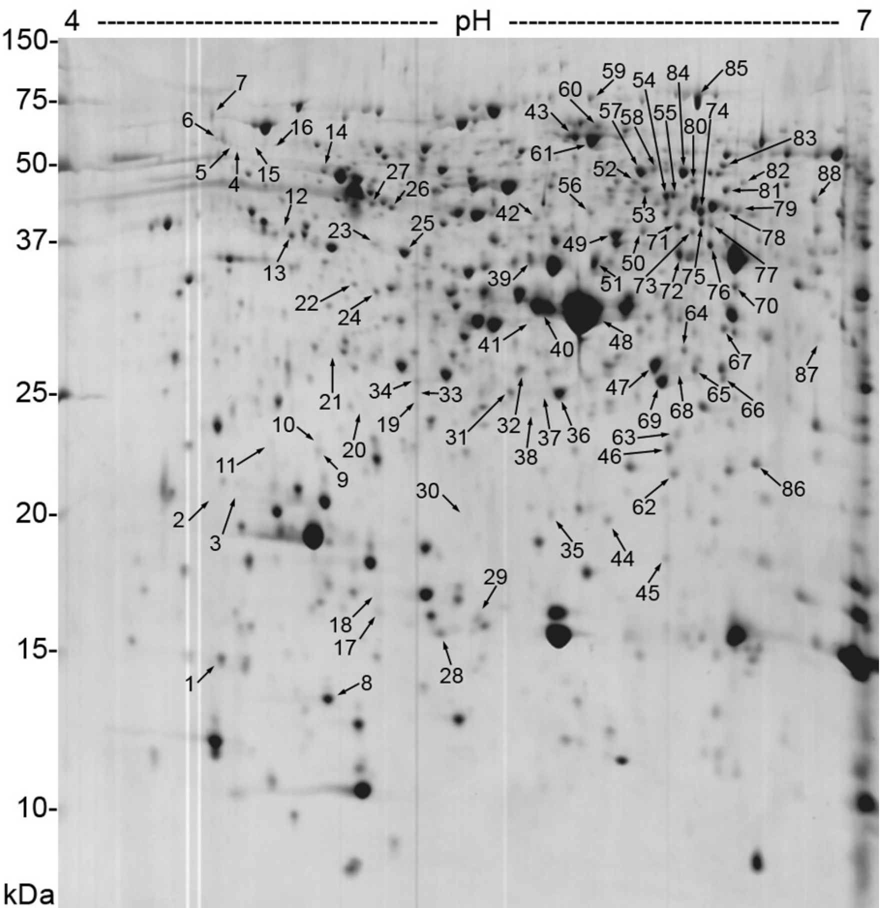

Figure 5-2. A representative 2-DE gel image of the sclerotial proteome of *S. sclerotiorum* during initiation. Samples containing 300 μg total protein were subjected to isoelectric focusing (IEF) in a linear IPG strip (17 cm, pH 4–7) as the first dimension, followed by 13% SDS-PAGE for protein separation in the second dimension. Proteins were visualized with Colloidal blue staining. The differentially abundant protein spots are indicated by arrows (the numbers refer to the order of each spot in Table 5-1).

Table 5-1. List of the differentially expressed proteins identified during sclerotial development of *S. sclerotiorum*

Spot [a]	Protein name [b]	Locus [c]	Accession no. [d]	Score [e]	PM [f]	Cov [g]	M_r/p*I* [h]	M_r/p*I* [i]	Pattern [j]
	Amino acid metabolism								
49	ketol-acid reductoisomerase, mitochondrial precursor	SS1G_03855	XP_001595766	637	22	40	44.2/8.78	38.0/6.06	0–3: a, b, b
51	L-threonine 3-dehydrogenase	SS1G_10495	XP_001588947	637	21	40	41.6/5.72	35.3/5.98	0–8: a, b, b
55	homogentisate 1,2-dioxygenase	SS1G_06873	XP_001592632	234	8	26	40.0/6.00	44.6/6.27	0–2: a, b, b
58	aldehyde dehydrogenase	BC1G_06362	XP_001554714	435	18	31	53.9/5.77	49.5/6.20	0–2: a, ab, b
64	hydroxyacylglutathione hydrolase	SS1G_00290	XP_001598204	293	11	38	33.8/6.19	28.5/6.31	0–3: a, b, b
65	NADP-dependent L-serine/ L-allo-threonine dehydrogenase ydfG	SS1G_13805	XP_001585237	421	8	40	29.0/6.24	27.2/6.35	0–3: a, b, b
74	S-adenosylmethionine synthetase	BC1G_04602	XP_001556886	541	15	36	43.3/5.91	41.8/6.37	0–3: a, b, b
71	S-(hydroxymethyl) glutathione dehydrogenase	SS1G_10135	XP_001588588	473	16	32	40.9/5.71	39.5/6.29	0–3: a, b, b
75	branched-chain-amino-acid aminotransferase	SS1G_12816	XP_001586239	497	18	33	41.5/6.18	40.0/6.37	0–3: a, b, c
77	bifunctional aspartokinase/homoserine dehydrogenase 2	SS1G_14424	XP_001584655	712	20	68	38.9/5.93	39.7/6.41	0–3: a, b, b
80	saccharopine dehydrogenase	SS1G_13213	XP_001585697	558	17	42	49.6/6.02	47.2/6.34	0–2: a, ab, b
82	saccharopine dehydrogenase	SS1G_13213	XP_001585697	227	8	22	49.6/6.02	47.0/6.53	0–3: a, b, b
83	methylmalonate-semialdehyde dehydrogenase, mitochondrial precursor	SS1G_04782	XP_001594974	931	20	41	61.9/8.47	50.2/645	0–3: a, b, b
84	aldehyde dehydrogenase	BC1G_06362	XP_001554714	644	27	40	53.9/5.77	48.9/6.31	0–3: a, b, c
	Carbohydrate metabolism								
4	cellobiose dehydrogenase	SS1G_13051	XP_001585959	629	26	41	60.8/4.53	56.3/4.70	0–12: b, b, a
5	cellobiose dehydrogenase	SS1G_13051	XP_001585959	448	22	24	60.8/4.53	56.5/4.67	0–5: b, b, a
6	cellobiose dehydrogenase	SS1G_13051	XP_001585959	511	20	34	60.8/4.53	59.4/4.60	0–7: b, b, a
15	cellobiose dehydrogenase	SS1G_13051	XP_001585959	510	22	29	60.8/4.53	57.2/4.74	0–22: b, b, a
16	cellobiose dehydrogenase	SS1G_13051	XP_001585959	468	20	27	60.8/4.53	59.1/4.81	0–20: b, b, a
24	D-arabinitol 2-dehydrogenase	SS1G_02446	XP_001596229	160	6	17	37.6/6.13	32.9/5.18	0–4: a, b, b
56	galactose-1-phosphate uridylyltransferase	SS1G_14008	XP_001585148	387	13	31	43.6/5.60	41.7/5.97	0–5: a, b, b
	Lipid and Secondary metabolism								
22	EstA	SS1G_01703	XP_001597509	429	15	49	35.5/5.14	33.5/5.10	0–4: a, b, b
50	diphosphomevalonate decarboxylase	SS1G_03958	XP_001595868	570	18	47	41.3/5.81	38.7/6.16	0–3: a, b, b

Table 5-1 Continuted

52	hydroxymethylglutaryl-CoA synthase	SS1G_14433	XP_001584664	570	22	46	51.0/5.89	47.0/6.13	0–5: a b b
76	sterol 24-C-methyltransferase	SS1G_03467	XP_001595378	568	16	43	42.3/6.07	38.8/6.41	0–4: a b b
	Nucleotide metabolism								
35	non-canonical purine NTP pyrophosphatase	SS1G_00208	XP_001598122	104	4	30	9.3/4.79	20.1/5.82	0–2: a b c
73	purine catabolism protein pucG	SS1G_10169	XP_001588622	423	15	37	41.8/6.02	38.7/6.34	0–3: a b b
	Energy								
1	ATP synthase delta chain, mitochondrial precursor	SS1G_07055	XP_001591609	109	5	24	17.7/5.43	14.9/4.62	0–3: a b c
14	ATP synthase subunit beta	SS1G_07793	XP_001591168	903	31	55	55.6/5.24	40.9/4.99	0–3: a b b
36	triosephosphate isomerase	SS1G_11433	XP_001587441	455	22	53	27.0/5.52	26.5/5.85	0–3: a b b
39	fructose-bisphosphate aldolase	SS1G_06561	XP_001592320	492	19	46	39.3/5.42	35.5/5.75	0–3: a b b
43	yvdP	SS1G_03616	XP_001595527	849	30	49	65.4/5.85	59.8/5.91	0–6: a b b
54	pyruvate dehydrogenase E1 component subunit alpha	SS1G_09626	XP_001588993	588	18	52	45.6/8.15	44.4/6.25	0–3: a b b
57	dihydrolipoyl dehydrogenase, mitochondrial precursor	SS1G_11008	XP_001587768	510	19	27	54.6/6.49	48.7/6.15	0–2: a b c
60	yvdP	SS1G_03616	XP_001595527	591	21	43	65.4/5.85	64.2/6.00	0–3: a b c
61	yvdP	SS1G_03616	XP_001595527	903	35	54	65.4/5.85	58.8/5.98	0–5: a b b
70	ribose-5-phosphate isomerase	SS1G_08408	XP_001590668	533	20	51	36.4/6.09	33.2/6.50	0–3: a b b
72	glyceraldehyde-3-phosphate dehydrogenase	SS1G_07798	XP_001591173	401	12	49	36.9/5.93	36.0/6.29	0–2: a b c
78	isocitrate dehydrogenase, mitochondrial precursor	SS1G_04924	XP_001593497	708	23	44	50.5/7.67	42.3/6.47	0–2: a b c
81	fumarate hydratase, mitochondrial precursor	SS1G_05243	XP_001593815	491	13	37	51.0/6.05	45.5/6.46	0–4: a b b
85	aconitate hydratase, mitochondrial precursor	SS1G_11047	XP_001587807	1023	27	34	85.7/6.26	73.7/6.36	0–4: a b b
	Transcription and Protein fate								
7	zinc finger protein zpr1	SS1G_02208	XP_001595992	655	22	45	56.2/4.58	69.3/4.58	0–23: a b b
12	small glutamine-rich tetratricopeptide repeat-containing protein A	SS1G_06491	XP_001592251	235	8	22	36.3/4.92	39.0/4.84	0–4: b b a
13	40S ribosomal protein S0	SS1G_12211	XP_001587181	323	12	41	32.0/4.76	38.1/4.87	0–2: a b b
42	26S proteasome regulatory subunit rpn-8	SS1G_01605	XP_001597411	715	23	55	39.6/5.42	40.7/5.77	0–3: a b c
53	elongation factor Tu	SS1G_00053	XP_001597967	536	16	42	49.5/6.38	45.5/6.17	0–3: a b c
62	proteasome endopeptidase complex chain PRE3	SS1G_12818	XP_001586241	320	9	64	16.7/5.41	21.8/6.27	0–2: a b b
88	RNP domain-containing protein	SS1G_02594	XP_001596374	550	17	57	38.2/6.74	43.8/6.79	0–7: a b b
	Cell Defense								

Table 5-1 Continuted

29	superoxide dismutase	SS1G_00699	XP_001598610	127	7	37	15.9/5.54	16.4/5.56	ab b a
44	glutathione peroxidase	SS1G_00741	XP_001598652	241	12	35	19.6/5.97	19.9/6.03	a b b
59	NADH-ubiquinone oxidoreductase subunit, mitochondrial precursor	SS1G_01767	XP_001597573	767	26	35	81.5/6.33	76.6/5.97	a b b
46	NADH:ubiquinone oxidoreductase 21.3kD	SS1G_03728	XP_001595639	443	17	68	21.3/6.43	22.9/6.25	a b c
63	NADH:ubiquinone oxidoreductase 21.3kD	SS1G_03728	XP_001595639	198	4	22	21.3/6.43	23.6/6.28	a b c
69	mitochondrial peroxiredoxin PRX1	SS1G_04704	XP_001594896	347	14	55	25.6/5.89	26.4/6.23	a b c
86	superoxide dismutase, mitochondrial precursor	SS1G_06246	XP_001593324	465	17	53	25.1/8.92	22.2/6.57	b b a
67	cytochrome c peroxidase, mitochondrial precursor	SS1G_12928	XP_001586350	501	19	43	40.8/9.01	30.0/6.46	a b b
	Differentiation								
45	conidial pigment biosynthesis scytalone dehydratase Arp1	SS1G_13314	XP_001585797	237	8	26	23.7/8.65	18.4/6.25	b a a
66	trihydroxynaphthalene reductase	SS1G_13315	XP_001585798	381	8	37	29.7/5.90	26.6/6.45	b a a
	Unknown								
2	conserved hypothetical protein	SS1G_06769	XP_001592528	232	10	7	121.7/4.70	20.8/4.62	b b a
3	conserved hypothetical protein	SS1G_14065	XP_001584968	136	7	20	35.2/5.74	21.1/4.70	b b a
8	conserved hypothetical protein	SS1G_03393	XP_001395304	155	7	27	15.2/6.29	13.4/5.01	a b b
9	conserved hypothetical protein	SS1G_14065	XP_001584968	189	9	27	35.2/5.74	22.8/4.97	b b a
10	conserved hypothetical protein	SS1G_14065	XP_001584968	165	9	25	35.2/5.74	23.4/4.97	b b a
11	conserved hypothetical protein	SS1G_14065	XP_001584968	184	7	20	35.2/5.74	22.0/4.81	c b a
17	conserved hypothetical protein	SS1G_14065	XP_001584968	135	5	16	35.2/5.74	16.5/5.20	b b a
18	conserved hypothetical protein	SS1G_14065	XP_001584968	126	4	10	35.2/5.74	17.1/5.19	c b a
19	conserved hypothetical protein	SS1G_14065	XP_001584968	235	10	34	35.2/5.74	24.9/5.33	b b a
20	conserved hypothetical protein	SS1G_14065	XP_001584968	222	10	30	35.2/5.74	24.5/5.15	c b a
21	conserved hypothetical protein	SS1G_06770	XP_001592529	529	15	30	52.9/4.89	27.9/5.03	b b a
23	predicted protein	SS1G_12133	XP_001587104	435	16	45	37.1/5.30	35.5/5.14	b b a
25	predicted protein	SS1G_12133	XP_001587104	632	28	57	37.1/5.30	34.7/5.27	b b a
26	predicted protein	SS1G_02331	XP_001596115	743	23	50	48.1/5.15	43.4/5.21	c b a
27	predicted protein	SS1G_02331	XP_001596115	804	26	59	48.1/5.15	43.5/5.11	b b a
28	predicted protein	SS1G_02331	XP_001596115	166	5	16	48.1/5.15	15.7/5.42	a b b

Table 5-1 Continuted

30	conserved hypothetical protein	SS1G_10397	XP_001588849	204	7	41	15.7/5.25	20.2/5.47	9; b, b, a
31	conserved hypothetical protein	SS1G_14065	XP_001584968	238	11	39	35.2/5.74	25.8/5.57	3; b, b, a
32	conserved hypothetical protein	SS1G_00871	XP_001598782	402	20	49	26.4/5.23	26.8/5.61	6; b, b, a
33	conserved hypothetical protein	SS1G_14065	XP_001584968	185	7	22	35.2/5.74	25.7/5.33	3; b, ab, a
34	conserved hypothetical protein	SS1G_14065	XP_001584968	301	13	38	35.2/5.74	26.4/5.33	4; b, b, a
37	conserved hypothetical protein	SS1G_14065	XP_001584968	347	18	47	35.2/5.74	26.4/5.77	5; b, b, a
38	HHE domain-containing protein	SS1G_09508	XP_001589786	140	5	26	21.7/5.48	24.5/5.62	50; b, b, a
40	conserved hypothetical protein	SS1G_14065	XP_001584968	734	35	70	35.2/5.74	31.8/5.79	2; c, b, a
41	conserved hypothetical protein	SS1G_14065	XP_001584968	446	19	49	35.2/5.74	30.1/5.76	4; b, b, a
47	conserved hypothetical protein	SS1G_07959	XP_001591333	357	15	43	29.6/5.99	27.4/6.20	3; a, b, b
48	conserved hypothetical protein	SS1G_14065	XP_001584968	509	25	58	35.2/5.74	31.5/5.97	2; c, b, a
68	conserved hypothetical protein	SS1G_14065	XP_001584968	368	14	39	35.2/5.74	27.1/6.29	2; c, b, a
79	conserved hypothetical protein	SS1G_01797	XP_001597603	315	12	36	45.5/6.19	42.0/6.51	4; a, b, b
87	conserved hypothetical protein	SS1G_14065	XP_001584968	596	21	59	35.2/5.74	30.1/6.73	8; b, b, a

[a] Respective identification number of spots as given on the 2-D gel image.

[b] Protein name based on BROAD database searching by locus tag in NCBI result

[c] Locus tag number in NCBI annotation

[d] Accession number in NCBI

[e] Mascot score (threshold score > 50)

[f] Number of peptide matched

[g] Percent sequence coverage (%)

[h] Theoretical molecular mass (M_r, kDa)/Isoelectric point (p*I*) value

[i] Observed molecular mass (M_r, kDa)/Isoelectric point (p*I*) value

[j] Temporal regulation patterns of identified proteins at three stages of sclerotial development. X-axes: from left to right is 4, 5, and 8 days of growth; Y-axes: relative abundance normalized as an expression ratio (expression ratio = 4 days/5 days and 8 days/5 days, with the expression ratio at 5 days set to one by default). The different letters indicate the significance as determined by ANOVA ($p < 0.05$).

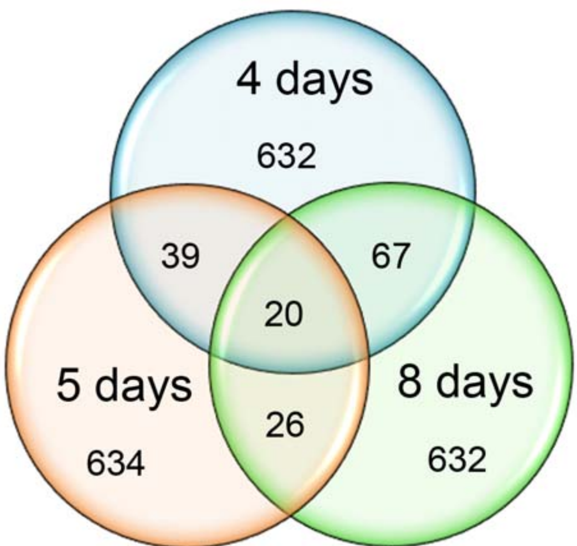

Figure 5-3. A Venn diagram showing the numerical distribution of protein spots during various stages of sclerotial development in *S. sclerotiorum* (4 days, initiation; 5 days, development; 8 days, maturation).

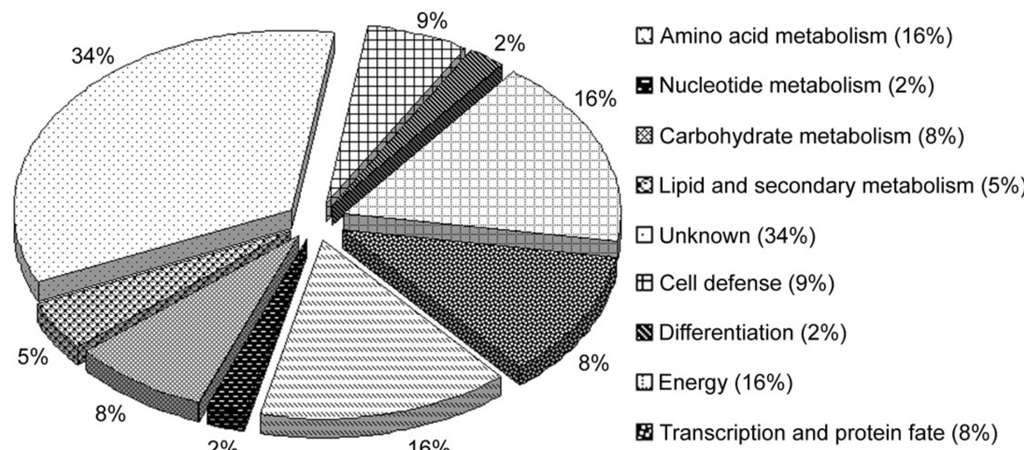

Figure 5-4. Functional classification of differentially abundant proteins identified during sclerotial development in *S. sclerotiorum*. The proteins were categorized based on their annotation in the InterPro (the European Bioinformatics Institute) and MIPS (Munich Information Center for Protein Sequences) database and assigned to their predicted functional categories.

mutant isolate of *S. sclerotiorum* was shown to be associated with aberrant sclerotial development and maturation (Rollins, 2003). Moreover, it has been speculated that lysine is involved in the binding between chitin and glucan during the synthesis of the fungal cell wall (Debono and Goedee, 1994). These observations indicate a possible crucial role for saccharopine dehydrogenase in sclerotial development, as well as a possible role in fungal virulence that will need to be tested in future studies.

The abundance of proteins involved in lipid and secondary metabolism was significantly higher at the initiation stage relative to the two later stages of sclerotial development (Table 5-1). For example, sterol 24-C-methyltransferase (spot 76) is involved in ergosterol biosynthesis, ergosterol being a component of fungal cell membranes (Hendrix, 1970; Nes et al., 2009). Similarly, diphosphomevalonate decarboxylase and hydroxymethylglutaryl-CoA synthase (spots 50 and 52, respectively) are known to participate in the biosynthesis of isoprenoids (Kleinig, 1998), which also represents an important, conserved set of reactions (Semighini et al., 2006). Metabolites derived from the sterol biosynthetic pathways often play significant roles as signaling molecules in regulating lipid synthesis (Edwards and Ericsson, 1999). Furthermore, the protein EstA (spot 22) has previously been used as an indicator of differentiation based on its isoenzyme patterns during sclerotial development in *Sclerotium rolfsii* (Chet and Henis, 1975).

Five spots identified as cellobiose dehydrogenase (spots 4, 5, 6, 15, and 16) and classified into the carbohydrate metabolism category were observed to be

significantly ($p < 0.01$) increased in abundance during the development and maturation of sclerotia, whilst the abundance of D-arabinitol 2-dehydrogenase and galactose-1-phosphate uridylyltransferase (spots 24 and 56, respectively) was significantly decreased in the latter two stages (Table 5-1). Cellobiose dehydrogenase (CDH) is an extracellular enzyme produced by a number of soft rot fungi and molds, and has been reported to exhibit high activity levels in the phytopathogen *S. rolfsii* (Ludwig and Haltroch, 2003).

CDH has also been reported to be involved in glucose oxidation and the production of superoxide (Henriksson et al., 2000). Interestingly, a correlation in abundance between CDH and superoxide dismutase (SOD; spots 29 and 86) was observed, and the latter also increased between the development and maturation stages (Table 5-1). SOD, a free radical scavenging enzyme, has been found to increase in abundance after differentiation during sclerotial metamorphosis (Georgiou et al., 2006). Another protein, peroxidase (spots 44 and 67), which was grouped in the cell defense category, exhibited a significant decrease between the initial and developmental stages. The abundance of catalase and polyphenoloxidase was not found to be significantly altered, although the occurrence of these enzymes has been reported in developing sclerotial extracts (Colotelo et al., 1971). Spots 46, 59 and 63 corresponded to NADH: ubiquinone oxidoreductase (complex I) of the fungal respiration chain, an enzyme known to generate superoxide radical ($O_2^{\cdot -}$) (Bai et al., 2003). Thus, enzymes involved in the generation and detoxification of free radicals appear to have an important role during sclerotial development.

The abundance of all proteins involved in energy metabolism decreased between the initiation and development stages of sclerotium formation. In fact, some protein spots (spots 1, 57, 60, and 72) decreased between the development and maturation stages as well (Table 5-1). Proteins in this category included isocitrate dehydrogenase, fumarate hydratase, and aconitate hydratase (spots 78, 81, and 85, respectively), which are enzymes involved in the citric acid cycle. Pyruvate dehydrogenase and dihydrolipoyl dehydrogenase (spots 54 and 57) are components of the pyruvate dehydrogenase complex that transforms pyruvate into acetyl-CoA, an important rate-limiting step for the entry of carbon into the citric acid cycle (Schnarrenberger and Martin, 2002). Ribose-5-phosphate isomerase (spot 70) is involved in the pentose phosphate pathway, which serves as a major source for NADPH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Walfridsson et al., 1995). In this study, GAPDH (spot 72) was most abundant during the sclerotial initiation stage, and gradually decreased during the developmental process. This finding is consistent with a previous study that reported a decrease in GAPDH activity during sclerotial development (Chet and Henis, 1975), further indicating an important role for this protein during the initial stages of sclerotial development.

Two proteins were identified as scytalone dehydratase and trihydroxynaphthalene reductase (spots 45 and 66, respectively), enzymes involved in the biosynthesis of melanin (1, 8-dihydroxynaphthalene, DHN) in a wide range of plant pathogenic fungi (Butler and Day, 1998). The abundance of these two proteins increased significantly between the initiation and

developmental stages of sclerotium formation, and was maintained at high levels after the initiation stage. This suggests that the up-regulation of proteins involved in melanin biosynthesis takes place during sclerotial development (Table 5-1). Inhibition of melanogenesis or degradation of melanin production could represent a potential strategy to mitigate the devastating effects of sclerotial pathogens. For instance, the white rot fungus *Phanerochaete chrysosporium* can degrade melanin produced by sclerotia of *S. sclerotiorum* (Butler et al., 2009).

Most proteins with unknown functions increased in abundance. All spots corresponding to one of these proteins, identified as a developmental-specific protein (Ssp1; SS1G_14065), were significantly increased between any two of the three stages of sclerotial development (Table 5-1). Some of these spots possessed a lower molecular mass than their theoretical/expected values, which may have resulted from fragmentation during the protein extraction process and/or electrophoresis (Horie et al., 2008). The rapid accumulation of Ssp has been observed only in sclerotial development, and it is undetectable in vegetative mycelia (Russo et al., 1982); in sclerotial extracts of *S. sclerotiorum*, *S. trifoliorum*, and *S. minor*, Ssp was found to comprise approximately 38, 27, and 31%, respectively, of the total cellular protein (Petersen et al., 1982). Therefore, this protein may have a role in the formation and preservation of dormancy or germination of sclerotia (Russo et al., 1982). Recently, Ssp1 was identified in multiple stages of sclerotial and apothecial development as a developmental-specific protein. Although the biological activity of Ssp1 is still not fully understood, the encoded gene product has been suggested as a biomarker for

sclerotial development (Li and Rollins, 2009). Further studies on the functional/structural characterization of Ssp1 should be undertaken to elucidate its biological significance, which could result in strategies to inhibit sclerotial development, thereby aiding in disease control. The characterization of other proteins with unknown functions may also provide insight into their roles, resulting in an increased understanding of the underlying mechanisms of sclerotial development. Ultimately, this knowledge may have applications towards the engineering of protection against sclerotial pathogens.

This study represents the first global analysis of the proteins associated with sclerotial development, and provides insight into the intrinsic regulatory networks driving this process. Some of the identified proteins may serve as rational targets for the development of novel and effective inhibitors of melanin synthesis, which could aid in plant protection by reducing sclerotium survival. In addition, proteins with unknown functions need to be characterized to elucidate their biological roles. As the data contained in publically available databases continue to expand, ascribing functions to the identified proteins becomes of greater importance in order to maximize the utility of such databases. Although the functions of many of the proteins found in this study might not be currently known, their association with sclerotial development offers some clues as to their putative activities. Indeed, the knowledge that these proteins are likely involved in sclerotial development is valuable, since it provides a context for future investigations on their function.

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6. The Proteome of liquid sclerotial exudates from *Sclerotinia sclerotiorum**

6.1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic, homothallic fungus belonging to the phylum Ascomycota (Bolton et al., 2006). More than 400 plant species, including economically important crops such as grain legumes, oilseeds, and various horticultural commodities, serve as hosts for this pathogen in many parts of the world (Boland and Hall, 1994). A common symptom of *S. sclerotiorum* infection includes the development of water-soaked lesions that eventually turn necrotic, while the growth of fluffy white mycelium on affected tissue is a common sign of infection. As suggested by the name of the pathogen, sclerotia play an important role in the disease and life cycles of *S. sclerotiorum*. Sclerotia can be found inside infected stem piths and also on the surfaces of infected plant tissues under favorable environmental conditions. The period of sclerotium formation coincides with the flowering and seed production stages of the host, which can therefore lead to the infestation of harvested seeds (Bolton et al., 2006). Sclerotia also serve as over-wintering, long-term survival and reproductive structures for the pathogen (Chet and Henis, 1975; Willetts and Bullock, 1992).

Sclerotia are formed under conditions that are unfavorable for vegetative growth and consist of three different layers: a thick-walled and pigmented rind, a thin-walled cortex, and a white medulla (Bardin and Huang, 2001). The presence of melanin in the rind is important for the survival and longevity of the

*A version of this chapter has been published. Liang, Y., Strelkov, S.E., Kav, N.N.V. 2010. J. Proteome Res. 9, 3290-3298.

sclerotium, helping to protect it from adverse environmental conditions and microbial degradation (Bell and Wheleler, 1986). Three distinct, overlapping stages have been described during sclerotial development: (1) initiation (aggregation of hyphae to form initials), (2) development (hyphal growth to full size), and (3) maturation (involving surface delimitation, pigmentation of peripheral rind cells and internal consolidation) (Townsend and Willetts, 1954).

The germination of sclerotia can be carpogenic (resulting in the formation of apothecia) or myceliogenic (resulting in the formation of mycelia) (Bardin and Huang, 2001). The nature of germination depends on the geographic origin of an isolate, temperature, and/or soil moisture conditions. When carpogenic germination occurs, apothecia form on a stipe at the soil surface, releasing millions of airborne ascospores. These ascospores can colonize senescent or necrotic tissues, including flower petals, which serve as a nutrient source after spore germination. In contrast, myceliogenic germination results in the production of hyphae that can directly infect plant tissue, and is associated with a melanin deficiency, the existence of melanin-inhibitors, and/or an absence of nutrients (Bardin and Huang, 2001; Hegedus and Rimmer, 2005).

The exudation of liquid droplets by sclerotia is a common feature during the early stages of sclerotial development (Cook, 1969), and many reports in the literature from the 1970s and earlier refer to such exudates. The accumulation of liquid droplets was observed on sclerotial surfaces when initials began to form, with the droplets increasing in size during the growth of the sclerotia (Cook, 1969). During maturation, the droplets gradually decreased in size and eventually

disappeared (Colotelo, 1974). The rapid loss of water as exudates during the early stages of sclerotial development may serve to maintain internal physiological balance by conversion and excretion of excess carbon compounds (Cook, 1969). The exudation may also result from the dehydration of cell walls and the polymerization of soluble compounds during the formation and thickening of sclerotial cell walls (Chet and Henis, 1975). The decreased moisture in tissues as a result of the active exudation of water and carbohydrates may have a significant influence on the long-term survival of the sclerotia (Willetts, 1971).

The chemical composition of the exudates is quite complex. Oxidized phenolics were found to accumulate in the exudates, giving them a clear to pale-yellow or dark-brown color (Willetts and Bullock, 1992). In addition, the exudates contain a number of soluble carbohydrates, including glucose, trehalose, inositol, and mannitol (Cook, 1969). Sclerotial exudates have also been reported to possess phenol oxidase activity, and to contain various salts, proteins and amino acids, but no catechol compounds (Jones, 1970). The chemical and physiological properties of the exudates have also been described, including analysis of dry mass, total protein, and crude lipid and ammonium content at different stages of development; in addition, the specific activities of various enzymes as well as the pH and fatty acid composition were investigated (Colotelo, 1973; Colotelo et al., 1973). However, to the best of our knowledge, there has been no detailed analysis of the proteins in the exudates. The exudate constituents are believed to be selectively re-absorbed into the sclerotia, resulting in the decrease in droplet volume (Colotelo, 1973; Colotelo et al., 1973).

Moreover, exudates were reported to effectively induce disease symptoms and to have deleterious effects on adjacent plant tissues, although these effects are not understood (Colotelo et al., 1973).

In this study, a proteomic analysis was performed in order to gain a better understanding of the nature of sclerotial exudates from *S. sclerotiorum*. The results are discussed within the context of sclerotial development as well as the disease and life cycles of the pathogen.

6.2. Materials and Methods

6.2.1. Growth conditions and collection of exudates

A mature sclerotium of wild-type *S. sclerotiorum* collected from an infected canola plant was sliced in half using a sterile scalpel and placed on potato dextrose agar (PDA; Becton Dickinson; Franklin Lakes, NJ, USA) in a Petri dish, with the sectioned surface in contact with the medium. The Petri dish was incubated at room temperature (22 ± 1 °C) for 4 days, until a colony formed around the sclerotium. A mycelial plug (5 mm) excised from the periphery of this young colony was placed on the center of another PDA plate and was incubated at room temperature for 5 days. Liquid exudates (droplets) were collected from the surfaces of developing, 5-day-old sclerotia using a sterilized Pasteur pipette with the aid of a stereomicroscope (6 × magnification) (Colotelo et al., 1971; Chen et al., 2004; Rollins and Dickman, 1998). Three individual sclerotia were originally used to start the mycelial cultures, from each of which approximately 50 plates of developing sclerotia served as the source of the exudate samples. Exudate

samples derived from each original sclerotium were considered to be one biological replication, and thus three independent biological replicates of exudate samples were prepared and analyzed by electrophoresis.

6.2.2. Protein preparation

Protein samples for electrophoresis were prepared as previously reported with some minor modifications as described below (Colotelo et al., 1971). Exudate droplets from the surfaces of developing sclerotia were collected and immediately filtered through a 0.22 μm filter unit (Millipore Corp., Bedford, MA, USA) and lyophilized overnight. The concentration of protein in the samples was determined using a modified Bradford assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard (Bradford, 1976).

6.2.3. 1-DE and 2-DE analysis

For one-dimensional gel electrophoresis (1-DE), protein samples (15 μg) dissolved in HPLC-water were mixed with 5× SDS-PAGE loading buffer [250 mM Tris-HCl, pH 6.8, 500 mM (dithiothreitol) DTT, 10% (v/v) SDS, 35% (v/v) glycerol, 0.05% (v/v) Bromophenol Blue] and placed in a boiling water bath for 5 min. Samples were applied to and separated on a 4–15% gradient Tris-HCl Ready gel (Bio-Rad) run in a Mini PROTEAN Cell (Bio-Rad) at 25 mA per gel for 30 min. Three biological replicates were analyzed in different wells of the same gel.

For two-dimensional gel electrophoresis (2-DE), liquid exudate was desalted by centrifugation through a Microcon YM-3 centrifugal filter unit (3,000 Da MWCO; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's

recommended procedure and lyophilized overnight to concentrate the protein. The protein samples were resolubilized in rehydration/sample buffer (Bio-Rad) containing 1% (v/v) tributylphosphine (TBP, Bio-Rad). Immobilized pH gradient (IPG) strips (7cm, pH 4–7, Bio-Rad) were passively rehydrated overnight with 50 μg protein in 125 μL rehydration/sample buffer. Isoelectric focusing (IEF) was performed using a PROTEAN IEF cell (Bio-Rad) programmed as follows: the voltage was first held at 250V for 15 min, then linearly increased from 250 to 4,000 V over 2 h, focused for 20,000Vh and held at 500V. The focused strip was equilibrated in 1 mL of equilibration solution [6 M urea, 2% (v/v) SDS, 20% (v/v) glycerol, and 0.37 M Tris-HCl, pH 8.8] containing 130 mM DTT with gentle agitation for 10 min twice, followed by another equilibration in 1 mL of equilibration buffer containing 135 mM iodoacetamide (IAA, Bio-Rad) for another 10 min twice. SDS-PAGE gels (13%, 1mm) were carried out in a Mini PROTEAN Cell at 25 mA per gel for 45 minutes. Two technical replicates of the electrophoresis were performed for each of three biological replicates.

Proteins were visualized by staining with a Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA, USA), followed by destaining in deionized water as per the manufacturer's instructions. Gel images were recorded on a GS-800 Calibrated Densitometer (Bio-Rad).

6.2.4. In-gel digestion of proteins

Protein bands (1 × 10 mm) were excised from the stained 1-DE gels using a Gridcutter (Gel Company, San Francisco, CA, USA), while additional gel slices representing major bands on 1-DE gels and protein spots on 2-DE gels were

excised with a sterile scalpel. The excised gel samples were placed in separate 1.5 mL microcentrifuge tubes for in-gel tryptic digestion, which was performed in a laminar flowhood. Briefly, gel samples were washed with 100 μL of HPLC-grade water (Fisher Scientific, Fair Lawn, NJ, USA) for 5 min, dehydrated with 50 μL of 100 % (v/v) acetonitrile (ACN) for 10 min, destained with 50 μL of 50 mM NH_4HCO_3/50% (v/v) ACN for 10 min (with vigorous intermittent vortexing), dehydrated with 50 μL of 100% (v/v) ACN for 10 min, and air-dried for 10 min. Proteins in the gel samples were reduced by incubation with 30 μL of 10 mM DTT/0.1 M NH_4HCO_3 at 56 °C for 30 min, which was followed by dehydration with 50 μL of 100% (v/v) ACN for 5 min. The samples were subsequently alkylated in 30 μL of 55 mM IAA/0.1 M NH_4HCO_3 at room temperature in darkness for 20 min. The samples were rinsed with 150 μL of 0.1 M NH_4HCO_3, dehydrated with 100% (v/v) ACN for 10 min, and air-dried, with the latter three steps repeated twice. Proteins present in the gel slices were digested with 30 μL of 0.02 μg/μL trypsin (Promega, Madison, WI, USA) in 40 mM NH_4HCO_3/10% (v/v) ACN, followed by incubation at room temperature for 60 min, and a subsequent incubation at 37 °C overnight. The trypsin digestion was terminated by adding 3 μL of 2% (v/v) formic acid (FA) and incubation for 30 min. The solutions were added to new microcentrifuge tubes and extracted twice with 15 μL of 50% (v/v) ACN/0.1% (v/v) FA. The extracts were thoroughly mixed and stored at –80 °C until MS/MS analysis.

6.2.5. LC-MS/MS

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of the proteins was performed in the Microarray and Proteomics Facility, Department of Biological Sciences, University of Alberta, Edmonton, Canada. Digested samples were analyzed in an Agilent 1100 Series LC/MSD Trap XCT ion trap mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) operated in the unique peptide scan auto-MS/MS mode. Each sample (20 μL) was injected using an autosampler and processed on a Zorbax 300SB-C18 (5 μm, 5 × 0.3 mm) column to concentrate the sample, followed by separation of the peptides on a Zorbax 300SB-C18 (5 μm, 150 × 0.3 mm) column. Gradient separation of the peptides was performed at a flow rate of 4 μL/min with a mobile phase consisting of solvent A [0.1% (v/v) FA in H_2O] and solvent B [0.1% (v/v) FA in ACN], under the following linear gradient programs: an initial mode of 3% solvent B for 5 min (0–5 min), to 15% solvent B for 3 min (5–8 min), to 45% solvent B for 42 min (8–50 min), to 90% solvent B for 10 min (50–60 min), to 3% solvent B for 1 min (60–61 min), and finally to 3% solvent B for 14 min (61–75 min) to clean the column prior to the application of the next sample. Peptide ion fragmentation data were collected by performing an MS 300-2000 m/z scan, followed by MS/MS analysis of the most intense ions. Raw spectral data were converted into Mascot Generic File (*.mgf) format using the default method in the ChemStation Data Analysis module.

6.2.6. Protein identification

All MS/MS spectra (*.mgf) were analyzed using the Mascot search engine (version 2.1; Matrix Science, MA, USA) and queried against the NCBI

nonredundant (NCBInr) database with fungi selected as the taxonomy. The following search criteria were used: one missed cleavage by trypsin allowed, fixed modification of carbamidomethyl, peptide tolerance of ±2.0 Da, MS/MS tolerance of ±0.8 Da, peptide charge of 1+, 2+, 3+, monoisotopic with no precursor, and ESI-TRAP instrument. Identified proteins were grouped into the different predicted functional categories based on InterPro and MIPS classifications (the Munich Information Center for Protein Sequences) (Apweiler et al., 2001; Puepp et al., 2004).

6.3. Results and Discussion

6.3.1. Formation of sclerotial exudates

Sclerotium formation and development of exudate droplets were consistent with previous reports (Colotelo, 1974). As the mycelium of wild-type *S. sclerotiorum* reached the outer edge of the Petri dish 3 days after inoculation, the colony was generally undifferentiated except for some areas of thicker growth (Fig. 6-1A). However, sclerotial initials were clearly visible by 4 days, at which time droplets of exudate began to develop and coalesce into larger droplets on the surface of the sclerotia (Fig. 6-1B). By 5 days after inoculation, the sclerotia became pigmented and the exudate droplets had enlarged considerably and could be readily observed with the naked eye (Fig. 6-1C and 6-1E). By 8 days, the exudate droplets had almost completely disappeared (Fig. 6-1D). Based on the relative abundance of exudates at 5 days, and earlier reports that the dry matter

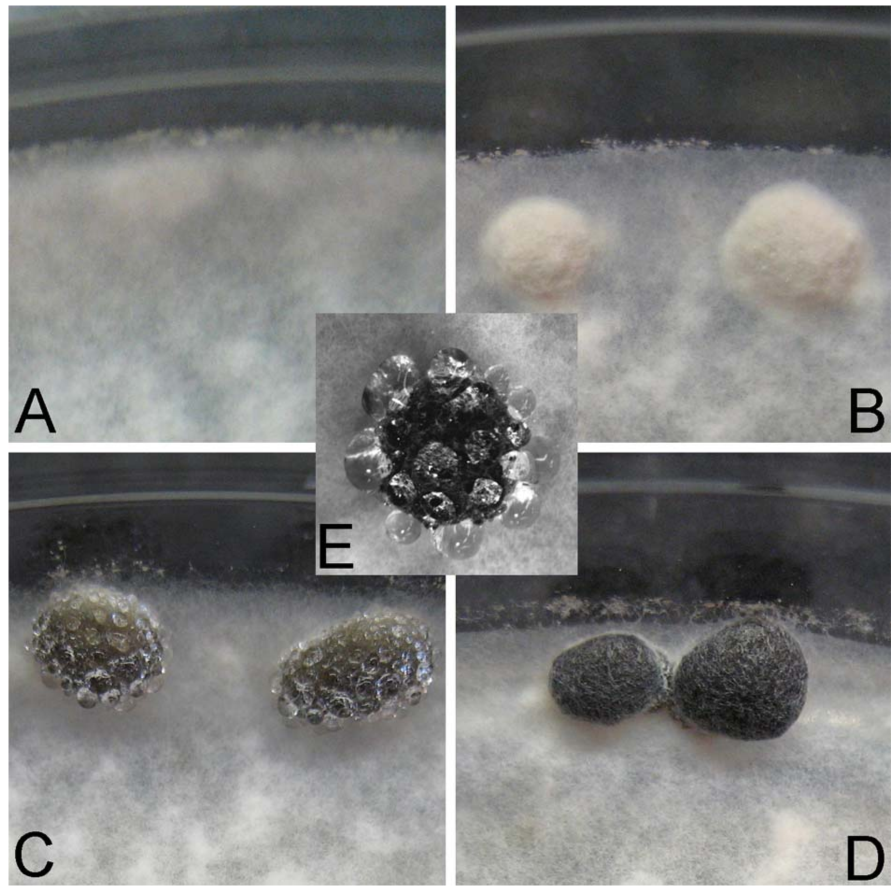

Figure 6-1. Formation of exudates by developing sclerotia of *S. sclerotiorum* at different developmental stages. (A) Mycelial mass 3 days after inoculation; (B) Sclerotial initials at 4 days after inoculation; (C) Developing sclerotia with visible exudate droplets at 5 days after inoculation; (D) Mature sclerotia at 8 days after inoculation; (E) Sclerotial exudates on the surface of the developing sclerotium under a stereomicroscope (25 × magnification).

and protein content of the exudates was greatest at this time, this time point was selected for collection of exudates for proteomic analysis (Colotelo, 1973).

6.3.2. Proteome of sclerotial exudates

The total protein concentration in the crude sclerotial exudate preparations was 2.3 ± 0.3 μg/μL (mean ± standard error), which is in consistent with previous reports (Colotelo, 1973). Both 1-DE and 2-DE were used in this study to ensure that the maximum number of sclerotial exudate proteins could be identified. 1-DE is technically-simpler than 2-DE and more importantly, its performance is not adversely affected by certain compounds in the exudates (e.g., phenolics and salts) as is normally the case with 2-DE. The attempts to separate proteins present in the crude exudates by 2-DE without any desalting process resulted in poor quality gels with considerable smearing of protein bands (data not shown). Therefore, additional steps to desalt and/or remove phenolics were required for 2-DE, but not for 1-DE. A combination of 1-DE and 2-DE was used to increase the chances of identifying proteins of lower abundance that would be missed in 2-DE, because the loss of such proteins may occur with additional desalting steps. However, the resolution of proteins is far superior in 2-DE, which separates proteins into individual spots on the gel. Furthermore, much lower molecular mass proteins could be observed and subsequently excised from 2-DE gels compared to 1-DE gels. Since 1-DE patterns obtained from each of the three independent biological replicates were identical, two gel panels, representing two independent biological replicates of sclerotial exudates, were further processed for mass spectrometric analysis. A total of 26 gel slices, corresponding to a

molecular mass range of 25-130 kDa, and four individually excised bands (Fig. 6-2), were subjected to tryptic digestion and LC-MS/MS for protein identification. In the case of 2-DE gels, 92protein spots reproducibly observed in all three biological replicates were excised and subsequently identified by LC-MS/MS. A representative gel image indicating the protein spots resolved by 2-DE is shown (Fig. 6-3).

6.3.3. Protein identification

LC-MS/MS analysis of proteins from excised gel spots/slices resulted in peptide mass fingerprints that were used to search the NCBInr database using Mascot software. Individual ion scores were calculated as $-10\times\text{Log}(P)$, where P is the probability that the observed match is a random event. Scores greater than the threshold value (> 50) were considered to indicate identity or extensive homology ($p < 0.05$), as defined by the Mascot probability analysis.

A total of 103 significantly positive protein hits, consisting of 50 nonredundant proteins, were reproducibly identified from the 1-DE gel slices (26 slices plus the 4 individual bands) and are shown in Table 6-1 with additional data presented in Table S6-1. The number of hits generated from each slice was roughly correlated with the intensities of the corresponding protein bands on the gels (Fig. 6-2). Multiple hits are a common occurrence in protein samples generated by 1-DE separation of crude protein extracts, likely as a result of the occurrence of more than one protein in a particular band. Moreover, some proteins were identified in more than one band, which may result from fragmentation during extraction and/or 1-DE (Table 6-1) (Horie et al., 2008). In

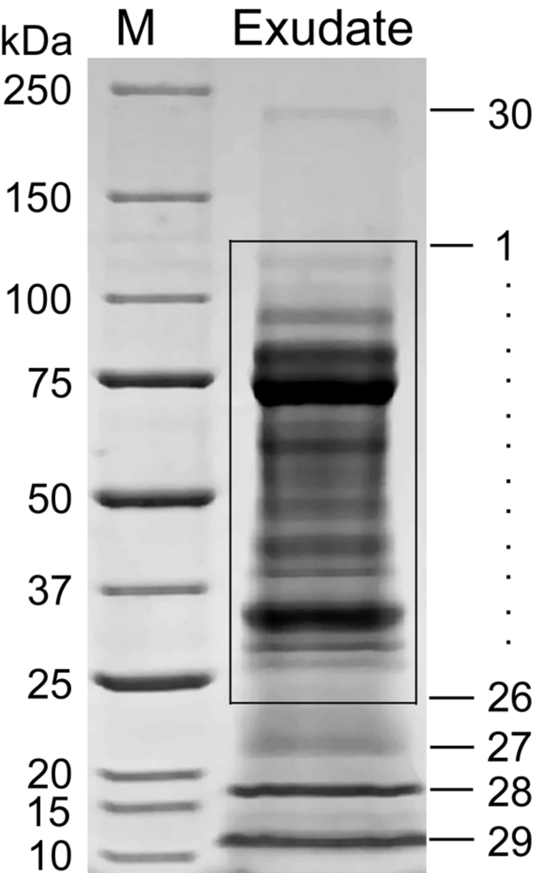

Figure 6-2. 1-DE analysis of proteins present in sclerotial exudates from *S. sclerotiorum*. Proteins were separated by 1-DE and visualized by staining with Colloidal Coomassie Blue. Bands of approximate 20-130 kDa in size (box) were cut into 26 (1 × 10 mm) slices (band nos. 1-26) and an additional four individual bands (band nos. 27-30) were excised for MS/MS analysis. The numbers ascribed to the bands on the gel correspond to the numbers and protein identities listed in Table 6-1. Relative migration of the molecular mass markers (M) is also indicated.

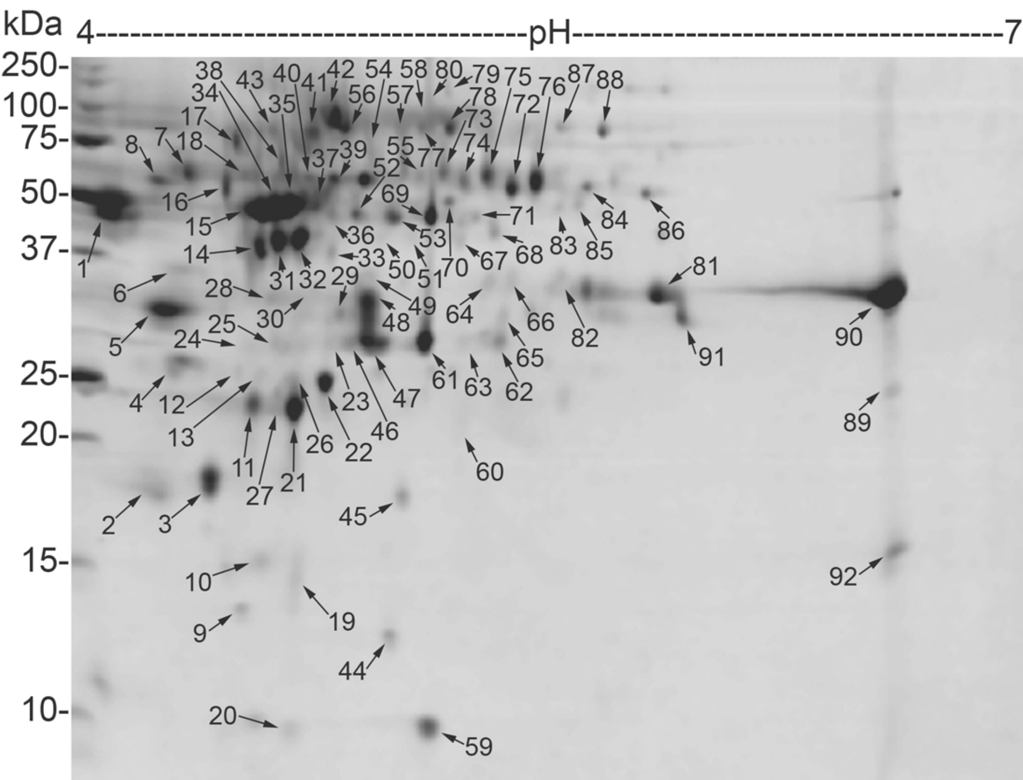

Figure 6-3. 2-DE analysis of proteins present in sclerotial exudates from *Sclerotinia sclerotiorum*. Proteins were separated by 2-DE and visualized by staining with Colloidal Coomassie Blue. The numbers ascribed to the spots on the representative gel image correspond to the numbers and protein identities listed in Table 6-2.

Table 6-1. List of proteins identified by 1-DE with LC-MS/MS in the sclerotial exudates of *S. sclerotiorum*

Band[a]	Protein name (function)[b]	Locus[c]	Accession no.[d]	Score[e]	PM[f]	Cov[g]	Mr /pI[h]
	Amino acid metabolism						
16	tyrosinase	SS1G_01576	XP_001597382	299/235	9/13	18/12	66.2/5.76
17	tyrosinase	SS1G_01576	XP_001597382	291/250	9/9	15/18	66.2/5.76
18	tyrosinase	SS1G_01576	XP_001597382	238/83	6/2	12/7	66.2/5.76
22	aspartate protease	SS1G_03181	XP_001595093	85/68	6/5	16/16	41.6/5.07
17	aspartyl protease	SS1G_03629	XP_001595540	143/105	4/4	16/13	44.9/5.80
19	tripeptidyl-peptidase 1 precursor	SS1G_07655	XP_001591030	136/92	4/5	6/9	61.9/4.97
20	tripeptidyl-peptidase 1 precursor	SS1G_07655	XP_001591030	116/58	4/3	8/8	61.9/4.97
13	leupeptin-inactivating enzyme 1 precursor	SS1G_07901	XP_001591275	195/99	7/3	18/14	55.2/4.78
06	glutaminase GtaA	SS1G_08889	XP_001590125	150/323	5/8	11/19	81.0/5.07
07	glutaminase GtaA	SS1G_08889	XP_001590125	534/271	14/8	27/17	81.0/5.07
20	gamma-glutamyltranspeptidase 2 precursor	SS1G_14127	XP_001584844	129/69	5/2	8/6	62.4/4.97
	Carbohydrate metabolism						
21	endoglucanase precursor	SS1G_00458	XP_001598370	187/199	4/6	12/24	40.9/4.25
22	endoglucanase precursor	SS1G_00458	XP_001598370	85/147	2/7	7/16	40.6/4.25
23	endoglucanase precursor	SS1G_00458	XP_001598370	53/78	1/3	5/10	40.6/4.25
06	beta-1,3-exoglucanase	SS1G_01229	XP_001597035	79/105	2/3	5/10	77.4/4.85
08	beta-1,3-exoglucanase	SS1G_01229	XP_001597035	395/295	16/9	28/19	77.4/4.85
09	beta-1,3-exoglucanase	SS1G_01229	XP_001597035	492/430	19/18	32/27	77.4/4.85
10	beta-1,3-exoglucanase	SS1G_01229	XP_001597035	318/287	9/8	20/21	77.4/4.85
11	beta-1,3-exoglucanase	SS1G_01229	XP_001597035	241/164	7/4	16/13	77.4/4.85
19	beta-1,3-exoglucanase	SS1G_01229	XP_001597035	88/103	2/3	7/10	77.4/4.85
30	beta-1,3-exoglucanase	SS1G_01229	XP_001597035	253/117	9/4	15/13	77.4/4.85
23	GH16_MLG1-glucanase	SS1G_01422	XP_001597228	76/190	2/7	8/25	34.5/5.59
15	alpha-amylase 1 precursor	SS1G_01776	XP_001597582	187/225	5/7	14/29	49.3/5.53
13	arabinofuranosidase/β-xylosidase precursor	SS1G_02462	XP_001596243	223/53	8/2	20/7	50.1/5.89
04	glyoxal oxidase	SS1G_02582	XP_001596362	179/99	4/4	8/8	82.7/5.66
05	glyoxal oxidase	SS1G_02582	XP_001596362	214/188	5/7	13/16	82.7/5.66
06	glyoxal oxidase	SS1G_02582	XP_001596362	111/202	2/8	3/14	82.7/5.66
10	glyoxal oxidase	SS1G_02582	XP_001596362	264/188	7/5	11/10	82.7/5.66
13	glyoxal oxidase	SS1G_02582	XP_001596362	160/150	3/5	4/11	82.7/5.66
07	alpha-N-arabinofuranosidase A precursor	SS1G_03602	XP_001595513	93/131	2/4	5/13	66.8/4.73
15	galactan 1,3-beta-galactosidase	SS1G_02618	XP_001596398	123/65	3/3	10/15	53.1/5.17
27	YoaJ	SS1G_04085	XP_001594278	194/69	5/2	23/15	30.9/4.48
03	alpha-1,2-mannosidase family protein	SS1G_04200	XP_001594393	247/146	9/6	17/15	85.0/4.74
04	alpha-1,2-mannosidase family protein	SS1G_04200	XP_001594393	519/358	18/15	27/22	85.0/4.74
05	alpha-1,2-mannosidase family protein	SS1G_04200	XP_001594393	365/188	9/7	1619	85.0/4.74
10	mannosidase MsdS	SS1G_04468	XP_001594660	197/209	6/6	17/23	58.1/4.66
11	mannosidase MsdS	SS1G_04468	XP_001594660	334/350	12/14	20/28	58.1/4.66
12	mannosidase MsdS	SS1G_04468	XP_001594660	352/270	10/10	25/28	58.1/4.66
13	mannosidase MsdS	SS1G_04468	XP_001594660	233/192	6/5	19/18	58.1/4.66
06	alpha-L-rhamnosidase A precursor	SS1G_04541	XP_001594733	287/161	9/3	18/6	78.1/5.04
07	alpha-L-rhamnosidase A precursor	SS1G_04541	XP_001594733	249/80	5/3	12/8	78.1/5.04
18	GPI-anchored cell wall beta-1,3-endoglucanase	SS1G_04852	XP_001593425	177/123	6/3	21/11	44.3/4.64
19	GPI-anchored cell wall beta-1,3-endoglucanase	SS1G_04852	XP_001593425	171/151	4/5	14/18	44.3/4.64
20	GPI-anchored cell wall beta-1,3-endoglucanase	SS1G_04852	XP_001593425	213/110	5/5	15/21	44.3/4.64
08	laccase precursor	SS1G_05112	XP_001593684	122/65	3/3	8/7	66.1/5.64
17	glucan 1,3-beta-glucosidase precursor	SS1G_06037	XP_001593115	239/127	5/4	11/	46.6/5.47
18	glucan 1,3-beta-glucosidase precursor	SS1G_06037	XP_001593115	196/208	6/9	21/14	46.6/5.47
02	beta-glucosidase 1 precursor	SS1G_07162	XP_001591716	201/61	6/2	10/4	94.2/5.55
04	1,3-beta glucanase	SS1G_07393	XP_001591947	53/230	2/8	4/19	83.9/4.79
05	1,3-beta glucanase	SS1G_07393	XP_001591947	325/431	12/16	20/30	83.9/4.79
06	1,3-beta glucanase	SS1G_07393	XP_001591947	658/647	22/25	30/37	83.9/4.79
07	1,3-beta glucanase	SS1G_07393	XP_001591947	641/366	19/12	30/28	83.9/4.79
08	1,3-beta glucanase	SS1G_07393	XP_001591947	148/163	4/5	10/14	83.9/4.79
09	1,3-beta glucanase	SS1G_07393	XP_001591947	260/181	7/6	15/14	83.9/4.79
01	cellobiose dehydrogenase	SS1G_07863	XP_001591237	88 /111	3/5	6/9	91.7/5.42
08	glucoamylase precursor	SS1G_08135	XP_001590395	123/115	3/3	12/11	59.3/4.71
09	glucoamylase precursor	SS1G_08135	XP_001590395	194/95	5/4	15/16	59.3/4.71
10	glucoamylase precursor	SS1G_08135	XP_001590395	152/101	4/4	13/16	59.3/4.71
17	1,3-beta-glucanosyltransferase gel1 precursor	SS1G_10353	XP_001588805	227/153	9/5	22/22	47.9/4.87
18	1,3-beta-glucanosyltransferase gel1 precursor	SS1G_10353	XP_001588805	298/215	10/7	21/22	47.9/4.87
19	1,3-beta-glucanosyltransferase gel1 precursor	SS1G_10353	XP_001588805	199/263	5/8	15/22	47.9/4.87
07	glucoamylase precursor	SS1G_10617	XP_001588171	257/229	7/7	21/20	72.5/5.39
08	glucoamylase precursor	SS1G_10617	XP_001588171	325/139	10/5	21/14	72.5/5.39
09	glucoamylase precursor	SS1G_10617	XP_001588171	130/126	3/4	9/11	72.5/5.39
10	1,3-beta-glucanosyltransferase gel4 precursor	SS1G_12017	XP_001586988	105/69	3/3	5/5	57.5/4.46
11	1,3-beta-glucanosyltransferase gel4 precursor	SS1G_12017	XP_001586988	112/89	5/4	5/5	57.5/4.46
12	1,3-beta-glucanosyltransferase gel4 precursor	SS1G_12017	XP_001586988	61/105	1/4	2/5	57.5/4.46
25	Necrosis inducing protein (NPP1)	SS1G_11912	XP_001586883	93/119	4/5	19/23	26.3/4.88
26	glucan 1,3-beta-glucosidase precursor	SS1G_12930	XP_001586352	122/89	4/3	23/18	32.0/4.25
	Energy						
27	malate dehydrogenase	SS1G_05337	XP_001593909	132/179	5/8	31/45	23.1/4.67
	Lipid and secondary metabolism						
29	esterase	SS1G_03299	XP_001595210	123/81	9/4	18/18	15.9/9.36

Table 6-1 Continuted

06	neutral ceramidase precursor	SS1G_04204	XP_001594397	261/146	7/6	13/15	73.8/5.20
07	neutral ceramidase precursor	SS1G_04204	XP_001594397	88/80	2/3	3/5	73.8/5.20
19	phosphatidylserine decarboxylase	SS1G_04563	XP_001594755	596/149	19/5	39/16	47.3/5.55
14	acid phosphatase precursor	SS1G_07639	XP_001591015	82/182	2/5	8/13	43.8/5.01
15	acid phosphatase precursor	SS1G_07639	XP_001591015	279/202	6/8	18/13	43.8/5.01
16	acid phosphatase precursor	SS1G_07639	XP_001591015	291/116	8/3	24/13	43.8/5.01
10	sphingomyelin phosphodiesterase precursor	SS1G_09965	XP_001589330	160/111	5/4	12/13	69.7/5.08
11	sphingomyelin phosphodiesterase precursor	SS1G_09965	XP_001589330	147/305	4/10	13/23	69.7/5.08
	Signal transduction						
23	FG-GAP repeat protein	SS1G_14133	XP_001584850	387/193	18/9	50/34	32.9/5.26
24	FG-GAP repeat protein	SS1G_14133	XP_001584850	353/296	15/21	50/50	32.9/5.26
	Unknown						
27	predicted protein	SS1G_00095	XP_001598009	96/83	4/3	19/36	17.7/5.10
17	predicted protein	SS1G_02331	XP_001596115	557/75	15/2	34/6	48.1/5.15
11	conserved protein	SS1G_04152	XP_001594345	109/116	3/6	11/20	57.6/5.00
12	conserved protein	SS1G_04152	XP_001594345	124/74	3/3	9/12	57.6/5.00
08	conserved hypothetical protein	SS1G_04473	XP_001594665	112/71	4/2	11/5	82.8/5.17
18	conserved hypothetical protein	SS1G_05304	XP_001593876	119/55	4/2	11/7	43.4/4.77
14	conserved hypothetical protein	SS1G_08645	XP_001590904	118/100	3/3	7/9	52.4/4.66
13	conserved hypothetical protein	SS1G_09143	XP_001589422	55/206	2/6	6/22	43.8/5.42
14	conserved hypothetical protein	SS1G_09143	XP_001589422	138/319	4/9	9/42	43.8/5.42
15	conserved hypothetical protein	SS1G_09143	XP_001589422	278/347	7/8	25/35	43.8/5.42
16	conserved hypothetical protein	SS1G_09143	XP_001589422	402/182	14/5	39/18	43.8/5.42
28	predicted protein	SS1G_09270	XP_001589549	183/98	5/4	23/16	18.1/4.64
28	predicted protein	SS1G_09838	XP_001589205	253/136	18/4	65/48	16.7/6.41
17	predicted protein	SS1G_10716	XP_001588269	123/56	3/2	14/11	31.5/4.45
18	predicted protein	SS1G_10716	XP_001588269	175/82	4/3	11/15	31.5/4.45
19	conserved hypothetical protein	SS1G_12917	XP_001586339	131/168	5/6	18/23	42.3/5.11
20	conserved hypothetical protein	SS1G_14065	XP_001584968	351/166	13/8	42/33	35.2/5.74
21	conserved hypothetical protein	SS1G_14065	XP_001584968	586/391	24/17	60/49	35.2/5.74
22	conserved hypothetical protein	SS1G_14065	XP_001584968	595/357	22/16	54/53	35.2/5.74
23	conserved hypothetical protein	SS1G_14065	XP_001584968	400/299	16/11	44/47	35.2/5.74
24	conserved hypothetical protein	SS1G_14065	XP_001584968	368/152	14/5	41/23	35.2/5.74
25	conserved hypothetical protein	SS1G_14065	XP_001584968	168/71	7/3	24/20	35.2/5.74

[a] Band number as given on the 1-D gel image.

[b] Protein name based on Broad database searching by Mascot results

[c] Locus tag number in BROAD database

[d] Accession number in NCBI database

[e] Mascot score (threshold score > 50, the different numbers separated by virgules represent results from biological replicates 1 and 2, respectively)

[f] Number of peptides matched (the different numbers separated by virgules represent results from biological replicates 1 and 2, respectively)

[g] Percent sequence coverage (%, the different numbers separated by virgules represent results from biological replicates 1 and 2, respectively)

[a] Theoretical molecular weight (Mr, kDa)/Isoelectric point (p*I*) value

addition, a total of 102 significantly positive protein hits, consisting of 42 nonredundant proteins, were identified from protein spots excised from 2-DE gels (Table 6-2 and Table S6-2). There are several multiple identifications from apparent single protein spots (e.g. spot 31, Table 6-2), possibly due to overlap of protein spots with similar molecular masses and isoelectric points (p*I*s). As indicated in the Venn diagram (Fig. 6-4), a total of 50 and 42 proteins were identified from 1-DE and 2-DE gels, respectively, with 36 of these proteins being identified from both types of electrophoresis. The value of performing both 1-DE and 2-DE is demonstrated by the fact that 14 proteins and 6 proteins were identified only from 1-DE and 2-DE gels, respectively. Clearly, if only one type of electrophoresis was performed, some proteins present in the sclerotial exudates would have been missed.

6.3.4. Functional categories

The identified proteins were classified into functional categories based on their known annotations in the currently available databases. These categories included amino acid metabolism, carbohydrate metabolism, lipid and secondary metabolism, energy, signal transduction, and unknown proteins (Fig. 6-5; Table 6-1 and Table 6-2). The largest category consisted of proteins involved in carbohydrate metabolism (45%) and included those proteins that are known to be related to the morphogenesis of the fungal cell wall. Fungal cell walls are composed of complex polysaccharides (glucan, chitin, mannans) and proteins (Bartnicki-Garcia, 1968; Wessels, 1994). The cell walls of *S. sclerotiorum* have

Table 6-2. List of proteins identified by 2-DE with LC-MS/MS in the sclerotial exudates of *S. sclerotiorum*

Spot[a]	Protein name (function)[b]	Locus[c]	Accession no.[d]	Score[e]	PM[f]	Cov[g]	Mr /pI[h]	Mr /pI[i]
	Amino acid metabolism							
53	tyrosinase	SS1G_01576	XP_001597382	364	18	21	66.2/5.76	44.9/5.09
55	tyrosinase	SS1G_01576	XP_001597382	136	5	12	66.2/5.76	58.9/5.18
67	tyrosinase	SS1G_01576	XP_001597382	141	8	12	66.2/5.76	38.9/5.32
69	tyrosinase	SS1G_01576	XP_001597382	266	11	15	66.2/5.76	54.1/5.22
70	tyrosinase	SS1G_01576	XP_001597382	63	2	5	66.2/5.76	49.2/5.28
71	tyrosinase	SS1G_01576	XP_001597382	184	9	15	66.2/5.76	45.0/5.37
64	aspartate protease	SS1G_03181	XP_001595093	80	3	19	41.7/5.07	33.6/5.41
31	aspartyl protease	SS1G_03629	XP_001595540	243	9	26	44.9/5.80	38.9/4.70
14	tripeptidyl-peptidase 1 precursor	SS1G_07655	XP_001591030	163	8	14	62.4/4.97	37.6/6.64
37	leupeptin-inactivating enzyme 1 precursor	SS1G_07901	XP_001591275	116	3	13	55.2/4.78	49.1/4.83
41	glutaminase GtaA	SS1G_08889	XP_001590125	454	15	29	81.0/5.07	81.1/4.82
43	glutaminase GtaA	SS1G_08889	XP_001590125	290	7	16	81.0/5.07	82.7/4.69
57	glutaminase GtaA	SS1G_08889	XP_001590125	286	9	17	81.0/5.07	83.0/5.12
77	glutaminase GtaA	SS1G_08889	XP_001590125	253	10	23	81.0/5.07	84.4/5.19
78	glutaminase GtaA	SS1G_08889	XP_001590125	491	16	32	81.0/5.07	84.5/5.28
76	gamma-glutamyltranspeptidase 2 precursor	SS1G_14127	XP_001584844	57	4	4	52.8/5.46	56.7/5.57
	Carbohydrate metabolism							
28	endoglucanase precursor	SS1G_00458	XP_001598370	195	7	26	40.9/4.25	32.0/4.69
30	endoglucanase precursor	SS1G_00458	XP_001598370	151	4	14	40.9/4.25	32.7/4.83
91	A chain A	SS1G_01422	XP_001597228	75	4	8	34.4/5.59	29.7/6.07
83	alpha-amylase 1 precursor	SS1G_01776	XP_001597582	100	3	10	49.3/5.53	47.5/5.65
84	arabinofuranosidase/ β-xylosidase precursor	SS1G_02462	XP_001596243	55	2	7	50.7/5.89	54.5/5.74
86	arabinofuranosidase/β-xylosidase precursor	SS1G_02462	XP_001596243	237	8	25	50.7/5.89	51.9/5.94
58	glyoxal oxidase	SS1G_02582	XP_001596362	329	12	17	82.7/5.66	98.9/5.15
72	glyoxal oxidase	SS1G_02582	XP_001596362	98	4	6	82.7/5.66	53.8/5.49
74	glyoxal oxidase	SS1G_02582	XP_001596362	133	4	7	82.7/5.66	56.7/5.33
76	glyoxal oxidase	SS1G_02582	XP_001596362	121	4	10	82.7/5.66	56.7/5.57
79	glyoxal oxidase	SS1G_02582	XP_001596362	475	19	24	82.7/5.66	97.6/5.35
80	glyoxal oxidase	SS1G_02582	XP_001596362	313	14	18	82.7/5.66	99.4/5.24
84	glyoxal oxidase	SS1G_02582	XP_001596362	419	15	24	82.7/5.66	54.5/5.74
36	galactan 1,3-beta-galactosidase	SS1G_02618	XP_001596398	173	5	19	53.1/5.17	45.4/4.89
52	galactan 1,3-beta-galactosidase	SS1G_02618	XP_001596398	351	11	41	53.1/5.17	45.8/4.97
56	alpha-N-arabinofuranosidase A precursor	SS1G_03602	XP_001595513	239	8	15	66.8/4.73	84.8/4.93
89	YoaJ	SS1G_04085	XP_001594278	136	3	16	31.3/4.48	23.7/6.77
42	alpha-1,2-mannosidase family protein	SS1G_04200	XP_001594393	532	18	24	85.0/4.74	85.1/4.90
1	mannosidase MsdS	SS1G_04468	XP_001594660	421	14	34	58.1/4.66	48.2/4.13
7	mannosidase MsdS	SS1G_04468	XP_001594660	286	7	22	58.1/4.66	60.3/4.40
8	mannosidase MsdS	SS1G_04468	XP_001594660	88	3	9	58.1/4.66	57.2/4.30
18	mannosidase MsdS	SS1G_04468	XP_001594660	220	9	24	58.1/4.66	59.1/4.63
39	mannosidase MsdS	SS1G_04468	XP_001594660	134	4	15	58.1/4.66	57.9/4.89
40	mannosidase MsdS	SS1G_04468	XP_001594660	210	7	15	58.1/4.66	58.6/4.81
73	mannosidase MsdS	SS1G_04468	XP_001594660	95	3	9	58.1/4.66	60.5/5.26
92	mannosidase MsdS	SS1G_04468	XP_001594660	279	6	22	58.1/4.66	15.6/6.79
43	alpha-L-rhamnosidase A precursor	SS1G_04541	XP_001594733	125	5	14	78.1/5.04	82.7/4.69
5	GPI-anchored cell wall beta-1,3-endoglucanase	SS1G_04852	XP_001593425	94	3	11	44.3/4.64	31.0/4.33
77	laccase precursor	SS1G_05112	XP_001593684	94	3	12	66.1/5.64	84.4/5.19
78	laccase precursor	SS1G_05112	XP_001593684	124	4	13	66.1/5.64	84.5/5.28
50	glucan 1,3-beta-glucosidase	SS1G_06037	XP_001593115	125	4	14	46.6/5.47	39.8/5.05
51	glucan 1,3-beta-glucosidase	SS1G_06037	XP_001593115	140	5	14	46.6/5.47	40.3/5.16
68	glucan 1,3-beta-glucosidase	SS1G_06037	XP_001593115	181	7	23	46.6/5.47	41.2/5.43
54	1,3-beta glucanase	SS1G_07393	XP_001591947	55	2	4	83.9/4.79	71.0/5.02
56	1,3-beta glucanase	SS1G_07393	XP_001591947	308	8	19	83.9/4.79	84.8/4.93
17	glucoamylase precursor	SS1G_08135	XP_001590395	352	12	30	59.3/4.71	71.6/4.56
6	1,3-beta-glucanosyltransferase gel1 precursor	SS1G_10353	XP_001588805	72	3	15	47.9/4.87	34.9/4.38
10	1,3-beta-glucanosyltransferase gel1 precursor	SS1G_10353	XP_001588805	96	4	15	47.9/4.87	15.1/4.65
14	1,3-beta-glucanosyltransferase gel1 precursor	SS1G_10353	XP_001588805	399	11	31	47.9/4.87	37.6/6.64
31	1,3-beta-glucanosyltransferase gel1 precursor	SS1G_10353	XP_001588805	340	12	28	47.9/4.87	38.9/4.70
13	necrosis inducing protein (NPP1)	SS1G_11912	XP_001586883	81	6	23	26.3/4.88	25.3/4.65
22	necrosis inducing protein (NPP1)	SS1G_11912	XP_001586883	122	6	17	26.3/4.88	24.5/4.86
26	necrosis inducing protein (NPP1)	SS1G_11912	XP_001586883	134	5	23	26.3/4.88	25.1/4.76
4	glucan 1,3-beta-glucosidase precursor	SS1G_12930	XP_001586352	223	8	33	32.0/4.25	26.4/4.38
12	glucan 1,3-beta-glucosidase precursor	SS1G_12930	XP_001586352	89	2	11	32.0/4.25	25.5/4.57
	Energy							
11	malate dehydrogenase	SS1G_05337	XP_001593909	207	13	45	23.3/4.67	22.6/4.62
21	malate dehydrogenase	SS1G_05337	XP_001593909	196	14	45	23.3/4.67	22.1/4.76
27	malate dehydrogenase	SS1G_05337	XP_001593909	121	5	45	23.3/4.67	23.5/4.70
	Lipid and secondary metabolism							
20	esterase	SS1G_03299	XP_001595210	93	7	20	15.9/9.36	9.6/4.74
59	esterase	SS1G_03299	XP_001595210	140	7	20	15.9/9.36	9.6/5.21
87	neutral ceramidase precursor	SS1G_04204	XP_001594397	360	14	22	73.8/5.20	85.3/5.66
88	neutral ceramidase precursor	SS1G_04204	XP_001594397	328	9	20	73.8/5.20	82.1/5.80
15	acid phosphatase precursor	SS1G_07639	XP_001591015	412	16	29	43.8/5.01	45.4/4.65
34	acid phosphatase precursor	SS1G_07639	XP_001591015	313	10	21	43.8/5.01	46.0/4.70
35	acid phosphatase precursor	SS1G_07639	XP_001591015	403	17	29	43.8/5.01	46.8/4.74
37	acid phosphatase precursor	SS1G_07639	XP_001591015	192	7	18	43.8/5.01	49.1/4.83
38	acid phosphatase precursor	SS1G_07639	XP_001591015	162	5	18	43.8/5.01	67.1/4.72

Table 6-2 Continued

9	allergen Asp f 15	SS1G_10096	XP_001588549	101	3	24	14.0/4.60	13.3/4.58
	Signal transduction							
23	FG-GAP repeat protein	SS1G_14133	XP_001584850	278	16	48	32.9/5.26	27.7/4.87
24	FG-GAP repeat protein	SS1G_14133	XP_001584850	184	11	29	32.9/5.26	27.8/4.70
25	FG-GAP repeat protein	SS1G_14133	XP_001584850	275	14	48	32.9/5.26	27.6/4.79
29	FG-GAP repeat protein	SS1G_14133	XP_001584850	139	5	29	32.9/5.26	30.4/4.91
32	FG-GAP repeat protein	SS1G_14133	XP_001584850	120	4	24	32.9/5.26	39.5/4.78
46	FG-GAP repeat protein	SS1G_14133	XP_001584850	344	21	52	32.9/5.26	27.7/4.94
47	FG-GAP repeat protein	SS1G_14133	XP_001584850	325	26	52	32.9/5.26	27.9/5.00
48	FG-GAP repeat protein	SS1G_14133	XP_001584850	363	39	61	32.9/5.26	31.4/5.00
49	FG-GAP repeat protein	SS1G_14133	XP_001584850	136	5	28	32.9/5.26	33.8/4.97
61	FG-GAP repeat protein	SS1G_14133	XP_001584850	429	46	60	32.9/5.26	27.9/5.20
62	FG-GAP repeat protein	SS1G_14133	XP_001584850	248	14	50	32.9/5.26	28.0/5.45
63	FG-GAP repeat protein	SS1G_14133	XP_001584850	147	5	28	32.9/5.26	28.1/5.34
	Unknown							
3	predicted protein	SS1G_00095	XP_001598009	75	2	19	17.7/5.10	18.2/4.47
45	conserved hypothetical protein	SS1G_02014	XP_001597818	121	5	15	43.1/5.19	17.5/5.12
44	conserved hypothetical protein	SS1G_03393	XP_001595304	174	8	39	15.2/6.29	12.4/5.08
16	conserved protein	SS1G_04152	XP_001594345	96	4	12	57.6/5.00	53.5/4.53
19	predicted protein	SS1G_04857	XP_001593430	100	4	35	15.0/4.56	14.3/4.76
37	conserved hypothetical protein	SS1G_07901	XP_001590904	81	3	9	52.4/4.66	49.1/4.83
2	conserved hypothetical protein	SS1G_08110	XP_001591483	94	3	17	19.4/4.52	17.5/4.30
75	conserved hypothetical protein	SS1G_09143	XP_001589422	148	5	20	43.8/5.42	59.2/5.41
85	conserved hypothetical protein	SS1G_09143	XP_001589422	212	8	22	43.8/5.42	48.4/5.72
60	conserved hypothetical protein	SS1G_09847	XP_001589214	140	4	19	22.0/5.05	18.7/5.32
33	conserved hypothetical protein	SS1G_12917	XP_001586339	135	7	24	43.1/5.11	36.7/4.88
65	conserved hypothetical protein	SS1G_12917	XP_001586339	132	6	18	43.1/5.11	30.4/5.46
66	conserved hypothetical protein	SS1G_14065	XP_001584968	90	4	19	35.2/5.74	34.2/5.49
81	conserved hypothetical protein	SS1G_14065	XP_001584968	340	19	47	35.2/5.74	32.2/5.98
82	conserved hypothetical protein	SS1G_14065	XP_001584968	225	10	44	35.2/5.74	33.2/5.62
90	conserved hypothetical protein	SS1G_14065	XP_001584968	545	22	54	35.2/5.74	32.2/6.77

[a] Respective identification number of spots as given on the 2-D gel image

[b] Protein name based on BROAD database searching by locus tag in NCBI result

[c] Locus tag number in NCBI annotation

[d] Accession number in NCBI

[e] Mascot score (threshold score>50)

[f] Number of peptides matched

[g] Percent sequence coverage (%)

[h] Theoretical molecular mass (Mr, kDa)/Isoelectric point (pI) value

[i] Observed molecular mass (Mr, kDa)/Isoelectric point (pI) value

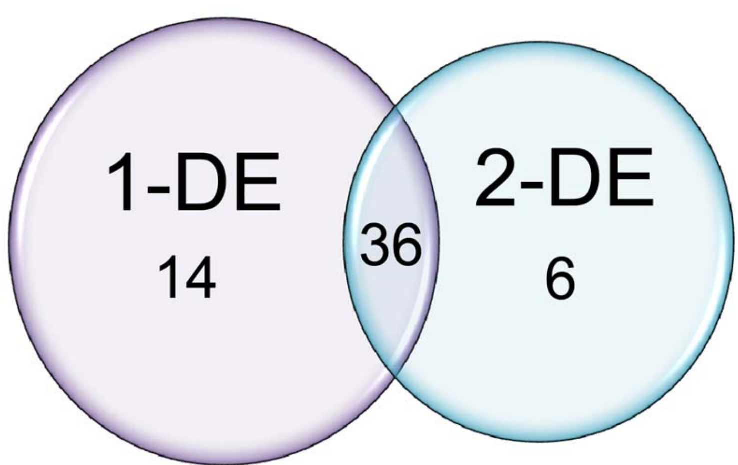

Figure 6-4. Distribution of the proteins identified using 1-DE and 2-DE coupled with LC-MS/MS. 1-DE and 2-DE independently identified 50 and 42 proteins, respectively, where 36 proteins were common in both datasets from the sclerotial exudates of *S. sclerotiorum.*

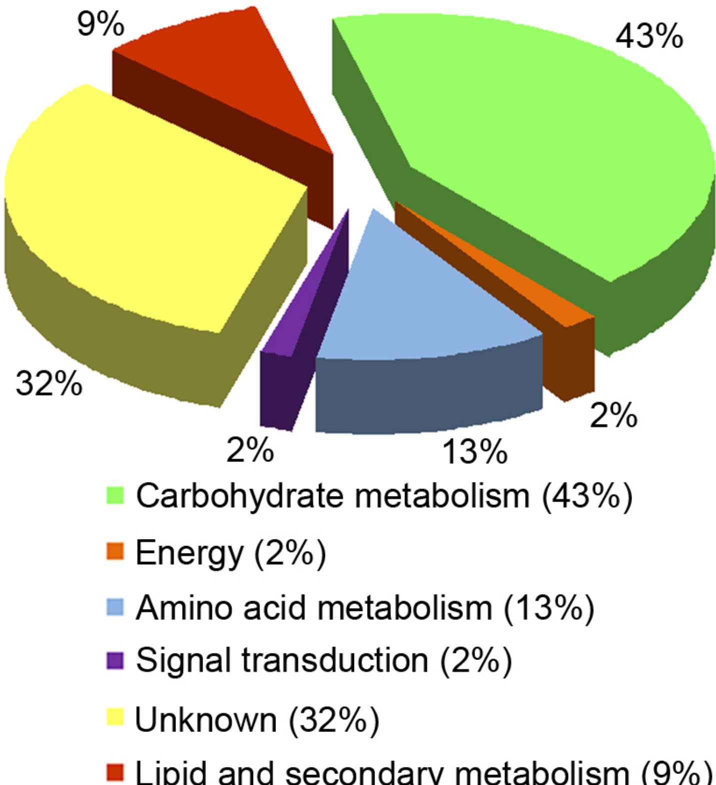

Figure 6-5. Functional groupings of proteins identified in sclerotial exudates from *Sclerotinia sclerotiorum*. The percentages listed correspond to the proportion of the identified proteins in the various functional categories, and are based on a set of 56 nonredundant proteins identified with unique accession numbers in the NCBI database.

been demonstrated to contain large amounts of 1,3-*β*-glucan and chitin (Jones, 1970), and most of the proteins related to cell walls have glucanase or chitinase activities (Adams, 2004). Five glucanases were identified from the sclerotial exudates (SS1G_00458, SS1G_01229, SS1G_01422, SS1G_04085, and SS1G_07393), and these enzymes may play important roles during early sclerotial development by modifying cell wall architecture. Necrosis inducing protein (NPP1, SS1G_11912) was also identified in the exudates. This protein is thought to mediate callose apposition, pathogenesis-related gene transcription, as well as the generation of reactive oxygen species (ROS) and ethylene in the plant response to abiotic and biotic stress (Fellbrich et al., 2002). Other proteins (SS1G_10353 and SS1G_12017) were reported to be responsible for the elongation of 1,3-*β*-glucan side chains (Hartland et al., 1996).

The localization of endoglucanase and glucanosyltransferase activities is dependent upon the expression of a GPI-anchor protein to a specific site in the fungal cell envelope (Adams, 2004). One such GPI-anchor protein (SS1G_04852) was also identified in the sclerotial exudates, which further supports an important role for cell wall modification enzymes during sclerotial development. Additional proteins identified in this study that have roles in modifying cell wall architecture included: glucoamylase, which is involved in hydrolysis of carbohydrates (SS1G_08135 and SS1G_10617) (Basaveswara et al., 1981), glycosyl hydrolase (SS1G_01776) (Lee et al., 2007), cellobiose dehydrogenase (SS1G_07863) (Sadana and Patil, 1985), mannosidase (SS1G_04200 and SS1G_04468) (Bartnicki-Garcia, 1968; Eades and Hintz,

2000), and rhamnosidase (SS1G_04541) (Bartnicki-Garcia, 1968; Orejas et al., 1999). Some of the other identified proteins (SS1G_06037, SS1G_07162, and SS1G_12930) have been reported to participate directly or indirectly in cell wall synthesis (Simons et al., 1998). A laccase (SS1G_05112) was also identified from the sclerotial exudates and has been shown to be associated with lignin degradation, morphogenesis, and pathogenic processes in numerous fungi (Thurston, 1994). A cell wall-bound laccase was also reported to be associated specifically with the process of melanization, through the oxidative polymerization of 1,8-dihydroxynaphthalene (Bell abd Wheeler, 1986).

Additionally, two proteins (SS1G_02462 and SS1G_03602) were identified as arabinofuranosidases (Table 6-1 and Table 6-2). An arabinofuranosidase precursor was previously identified in the secretome of *S. sclerotiorum* (Yajima and Kav, 2006), and has recently been demonstrated to be a virulence factor for this fungus (Yajima et al., 2009). The observation that sclerotial exudates may induce disease-like symptoms on plant tissues may reflect the presence of cell wall degrading enzymes (CWDEs) (such as the arabinofuranosidase) in the exudates (Colotelo, 1973). A protein (SS1G_10096) belonging to the cerato-platanin (CP) family was also identified. The CP family includes phytotoxic proteins that are secreted by the Ascomycetes and which elicit defense-related responses, including phytoalexin synthesis and cell death, in host and nonhost plants (Pazzagli et al., 2006). The potential roles of these proteins, as well as others, during exudate formation and sclerotial development will be the subject of future investigations.

Another major group of proteins identified in this study include those involved in amino acid metabolism (13%), such as tyrosinase (SS1G_01576), which has been shown to be a key enzyme in the biosynthesis of fungal melanin (Bell and Wheeler, 1986). There is considerable information in the literature that suggests that melanin is essential for fungal survival and longevity, by affording protection against adverse environmental stresses including UV irradiation, desiccation, antagonistic microbes, nutrient limitation, and temperature extremes (Bell and Wheeler, 1986; Whiletts, 1971; Henson et al., 1999). Furthermore, the presence of melanin, which is known to act as a free radical scavenger, may indirectly indicate that sclerotial differentiation processes can be induced by oxidative stress.

Most of the remaining proteins were classified as unknown (32%), as they were not functionally annotated in the public databases. One particularly abundant protein (SS1G_14065) has been reported to be a development-specific protein (Ssp1) in multiple stages of sclerotial development, although its biological activity is unknown at this time (Li and Rollins, 2009); the same protein has been shown to rapidly accumulate during sclerotial development, but has not been detected in vegetative hyphae (Russo et al., 1982). This protein was also shown to comprise approximately 38% of the total protein in sclerotial extracts, and is suggested to play significant roles in sclerotial development in other *Sclerotinia* species (Petersen et al., 1982). Structural and functional characterization of Ssp1 may allow for the elucidation of its function during sclerotial development and will be pursued further. The development of inhibitors of this and other proteins

(such as arabinofuranosidase) may also be an effective strategy to inhibit sclerotial development and may have applications in disease management.

6.4. Concluding remarks

The effectiveness of proteome-level investigations in the study of plant pathology is supported by the identification of potential proteins that may be involved in pathogen development and/or pathogenicity and virulence. The objective of the current study was to evaluate the protein composition of the liquid exudates formed during sclerotial development in *S. sclerotiorum*. The results obtained provide, for the first time, a comprehensive profile of the proteins present in these exudates, which will form the basis for investigations into the structure and function of selected proteins in sclerotial development. Amongst the proteins identified was arabinofuranosidase, which has been previously demonstrated to be a virulence factor for *S. sclerotiorum* (Yajima and Kav, 2009). This finding suggests an additional role for this enzyme during sclerotial development, which has not been previously proposed. Another protein (SS1G_14065) identified in the exudates has been shown to be present in large amounts only in the developing sclerotia (Li and Rollins, 2009; Russo et al., 1982). This protein could also serve as a target for the development of novel crop protection agents. A number of proteins with unknown functions were also identified in this study; characterization of their roles during sclerotial development, and in the life cycle of *S. sclerotiorum* in general, should form the focus of future studies.

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7. General Discussion

The two main objectives of this project were (1) to gain a comprehensive understanding of the response of canola to challenge by *S. sclerotiorum*, and (2) to characterize some of the main biological, physiological, and molecular changes associated with growth and infection by this fungus. In order to achieve these objectives, a series of proteome-level investigations were conducted. These included an analysis of proteome-level changes in canola inoculated with *S. sclerotiorum* and/or treated with the fungal pathogenicity factor oxalic acid, as well as characterization of the sclerotial proteome. Additionally, the signal transduction networks (e.g., ABA, ET, JA, SA) and expression and/or activity of enzymes (e.g., CAT, NOX, OXO, POX, SOD) involved in the host oxidative response were examined, as were some of the morphological and histological events associated with infection. Finally, the role of a protein, termed Sssp, with unknown function was evaluated via a gene-disruption approach. It was hoped that, collectively, this research would be useful not only in furthering the understanding of the canola/*S. sclerotiorum* interaction, but that it would also generate novel insights that might lead to the development of improved disease management strategies.

The comparative proteome-level analysis of the canola-*S. sclerotiorum* interaction (Chapter 2) led to the identification of a number of proteins for which the corresponding genes were not found to be differentially expressed in a previous, transcriptome-based analysis of the same interaction (Yang et al., 2007). It is clear that, in order to fully understand the canola-*S. sclerotiorum* relationship

or other important pathosystems, information must be obtained at several levels, since quantities of transcripts and proteins are often not well-correlated (Gygi et al., 1999). Moreover, since the studies described in this dissertation employed a susceptible canola genotype as the host, further studies should examine the interaction between resistant and tolerant genotypes and *S. sclerotiorum*. These additional studies would serve to complement the present work and provide more clues as to the basis for resistance to stem rot.

The use of biotechnology and genetic engineering approaches represents one avenue for obtaining durable disease resistance in economically important crops. Defense pathways, resistance genes and/or the hypersensitive response have all been suggested as rational targets for such an approach (Stuiver and Jerome, 2001). Given the role of OA as a pathogenicity factor for *S. sclerotiorum*, the manipulation of OXO (an enzyme that degrades OA) may represent an effective way in which to improve resistance to this pathogen (Thompson et al., 1995). Overexpression of oxalate decarboxylase, another enzyme that degrades OA, was shown to enhance resistance to *S. sclerotiorum* in tobacco and tomato (Kesarwani et al., 2000). Such reports suggest that interference with the production and/or accumulation of fungal pathogenicity or virulence factors could be sufficient to provide resistance, at least in *S. sclerotiorum* and other OA-secreting pathogens (Stuiver and Jerome, 2001).

Within this context, the current research provided novel insights that could be of use in transgenic-based approaches for obtaining resistance against *S. sclerotiorum*. For instance, we demonstrated the differential abundance of

molecules associated with JA-mediated signal transduction in response to *S. sclerotiorum* infection (Chapter 2 and 3). This finding suggests that the modulation of key genes in the JA pathway could lead to the development of crops tolerant to this fungus or other OA-secreting pathogens. Similarly, we observed that the abundance of a number of key enzymes involved in the host oxidative response, namely POX and SOD, was significantly reduced by treatment with OA (Chapter 3). This would indicate that over-expression of these proteins in *B. napus* might serve to mitigate the effect of OA, and/or modulate oxidative responses in general. Perhaps one of the best candidates identified in this study for the plantibody engineering of resistance to *S. sclerotiorum*, however, is the protein Sssp, since we showed that it serves as a virulence factor for the fungus (Chapter 4). As the role of Sssp was formerly completely uncharacterized, manipulation of this protein would represent an entirely novel approach for developing *S. sclerotiorum*-resistant canola.

In a knowledge-based approach to plant disease management, understanding the biology and development of a pathogen may be just as important as understanding the nature of its interactions with host genotypes. As such, a portion of this dissertation was dedicated to characterizing the process of sclerotial development in *S. sclerotiorum* (Chapter 5 and 6). An increased understanding of this important process could eventually result in new, fungus-specific methods of stem rot control, focused on the inhibition of sclerotium formation (Patsoukis and Georgiou, 2007). This could represent an attractive alternative to the traditional use of foliar fungicides. More generally, however,

an enhanced knowledge of the molecular regulation of sclerotial development could also be very important for understanding more complex forms of differentiation in other microbes. In this proteomic analysis of sclerotial development, we identified 88 differentially abundant proteins at different time-points, including development-specific protein (Ssp) which could have an important role in sclerotial development. This analysis represented the first global investigation of the sclerotial proteome, and sets the basis for future studies examining specific proteins with unknown functions during sclerotial development.

One of the problems associated with determining the function(s) of hypothetical or predicted proteins identified in global genomic or proteomic studies is that it may be difficult to determine in which, if any, biological processes a particular protein is involved. Ultimately, this necessitates a comprehensive approach. The work noted above on Sssp (Chapter 4) represents the integration of genomic, proteomic and transcriptomic analyses to develop specific hypotheses and identify targets for further analysis; in the case of Sssp, these analyses consisted of gene-disruption studies coupled with pathogenicity assays on canola plants. The identification of Sssp as a target for such studies would not have been possible without the proteome-based research that preceded this work, or without the comparison to the Genbank databases.

Given that *Sssp* gene-disruption mutants caused significantly reduced symptoms on a susceptible canola genotype, and that this protein is therefore a virulence factor for *S. sclerotiorum*, it worth touching on some possible

mechanisms of Sssp action. For instance, Sssp might interfere with the function or activities of cell-wall degrading enzymes, or it could possibly have a direct effect on the integrity of plant cell walls. Alternatively, Sssp might serve to attenuate the host defense response. Based on current knowledge, all of these possibilities are simple speculation, but could serve as the basis for more detailed studies on the properties of Sssp and its specific role in disease development. Moreover, Sssp could also be used as a target in an antibody or PCR-based diagnostic assay for *S. sclerotiorum* and/or *B. cinerea*, given the unique occurrence of this protein in these pathogens (Chapter 4). Such an approach would complement existing disease forecasting systems, and could facilitate development of in-field diagnostic tools in a variety of formats.

7.1. Concluding remarks

The proteins identified in this dissertation represent novel information in the understanding of the canola/*S. sclerotiorum* interaction and in the developmental biology of the fungus itself. Therefore, this analysis may serve as a starting point towards achieving a comprehensive understanding of the molecular events associated with stem rot disease specifically, and other host-pathogen interactions in general. Ultimately, an improved knowledge of stem rot and other diseases may lead to the creation of rational and effective strategies for sustainable plant disease management. Before such strategies can be implemented, however, additional work will be required. For instance, the function of some of the differentially abundant proteins identified in Chapters 2 and 3 should be studied in

more detail through gene knock-out or silencing studies. Similarly, while we gained insights into the signaling and oxidative responses of canola to *S. sclerotiorum* infection, these components could be examined more closely through additional molecular and histopathological analyses.

In the long-term, the modulation of host genes/proteins involved in the response to *S. sclerotiorum* challenge, through the application of various molecular techniques such as gene overexpression and RNA interference, could be useful for the management of stem rot. Specifically, the triggering of signaling pathways or the modulation of oxidative responses early in the infection process could serve to increase tolerance to *S. sclerotiorum*. Perhaps the clearest target for such an approach identified in this dissertation is the Sssp protein, which was found to serve as a virulence factor for *S. sclerotiorum* (Chapter 4). The expression of exogenous antibodies specific for this protein in canola might lead to increased tolerance/resistance to infection. Of course, it would be prudent to fully characterize the role of Sssp in disease development prior to undertaking such a strategy. Nevertheless, given the unique occurrence of Sssp in *S. sclerotiorum* and *Botrytis cinerea*, this protein could at the very least serve as a target for molecular detection of stem rot through an antibody or PCR-based approach.

While Chapters 2 to 4 were focused on the interaction between *S. sclerotiorum* and its canola host, the characterization of proteins associated with sclerotium development (Chapter 5) and sclerotial exudates (Chapter 6) represented a contribution to our understanding of the fungus itself. Ultimately,

such an understanding could also have important practical applications. By specifically targeting particular proteins or processes within these structures, it may be possible to hinder their formation and/or persistence. As such, the inoculum potential in canola fields could be reduced. While such strategies may seem a long way off, improved understanding of the basic biological systems involved is necessary before they are even possible. This dissertation has contributed to such an understanding, providing a good overview of the stem rot pathosystem and a strong foundation from which to build on our knowledge. Ultimately, a more comprehensive knowledge base will facilitate management not only of *S. sclerotiorum*, but also of related pathogens.

7.2. Literature cited

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Supplementary data

Table S5-1. Detailed mass spectrometric data of proteins identified during development of sclerotia of *S. sclerotiorum*

Spot[a]	Accession no.[b]	MS/MS Peptide sequence (charge)[c]
1	XP_001591609	K.LSLALPHQSVYR.S (3+) K.IVGGSGSEQDIAEAK.I (2+) K.IELEVLETLQAHLK.- (3+)
2	XP_001592528	R.SAWPPDTER.T (2+) R.TEPEEPMDGGIFEDGYWTDPPR.E (3+) K.ITSVEQLR.D (2+) K.YGQQLGSFYDVIATIK.D (2+) K.RAPLVPFISDAGLDMK.K (3+) R.APLVPFISDAGLDMK.K (2+) K.KFEVGFK.S (2+) K.FEVGFK.S (2+)
3	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (3+) K.DANFDNQPSIHLLYMNK.D (3+) R.MKGDNPIWEDAK.V (3+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (3+)
4	XP_001585959	R.LYDTQPSTSLTSQDGIR.Y (2+) R.YLQSGYNAAR.K (2+) R.KWLVEGLGYK.D (2+) K.WLVEGLGYK.D (2+) K.ADDKTGVFGYPNFFYSDGER.G (3+) K.TGVFGYPNFFYSDGER.G (2+) R.GGPVTTYLQSSLQR.S (3+) R.HGDTATGVTALVNGVETFISVTPTGR.V (3+) R.VILSAGAIISPSLLMHSGIGDLATLSR.L (3+) K.YSYESPPPEDR.D (2+) K.YSYESPPPEDRDLYLNSR.S (3+) R.SGPYTFASETSVFWTTISHADGTK.A (2+) R.EEIETYITTWSAYAR.G (2+) R.GQVNHWSSSCR.L (3+) K.VVGTTNLHVVDASIIAPVTVNPQFAVMAAAEK.A (3+) K.AAELILK.S (2+)
5	XP_001585959	R.LYDTQPSTSLTSQDGIR.Y (2+) R.YLQSGYNAAR.K (2+) R.KWLVEGLGYK.D (2+) K.WLVEGLGYK.D (2+) K.WLVEGLGYKDVDINDK.A (3+) K.DVDINDKADDK.T (3+) K.ADDKTGVFGYPNFFYSDGER.G (3+) K.TGVFGYPNFFYSDGER.G (2+) R.GGPVTTYLQSSLQR.S (2+) R.HGDTATGVTALVNGVETFISVTPTGR.V (3+) K.YSYESPPPEDRDLYLNSR.S (3+) R.DLYLNSR.S (2+) R.SGPYTFASETSVFWTTISHADGTK.A (3+) R.GQVNHWSSSCR.L (2+) K.VVGTTNLHVVDASIIAPVTVNPQFAVMAAAEK.A (3+) K.AAELILK.S (2+)
6	XP_001585959	R.LYDTQPSTSLTSQDGIR.Y (3+) R.YLQSGYNAAR.K (2+) R.KWLVEGLGYK.D (2+) K.WLVEGLGYK.D (2+) K.TGVFGYPNFFYSDGER.G (2+) R.GGPVTTYLQSSLQR.S (2+) R.HGDTATGVTALVNGVETFISVTPTGR.V (3+) R.VILSAGAIISPSLLMHSGIGDLATLSR.L (3+) K.YSYESPPPEDR.D (2+) K.YSYESPPPEDRDLYLNSR.S (3+) R.SGPYTFASETSVFWTTISHADGTK.A (3+) R.EEIETYITTWSAYAR.G (2+) R.GQVNHWSSSCR.L (3+) K.AAELILK.S (2+)
7	XP_001595992	K.DGTVTNGALESDEDQR.V (3+) R.VVDEIESLCMNCHENGITR.L (3+) R.IPYFR.E (2+) R.LTTPEDFSR.Q (2+) K.FIELDLEVPEGR.G (3+) K.EQIPEVYEK.I (2+) K.IEDVITR.G (2+) K.MLAGEAFPFR.L (2+) R.LSLDDPAGNSWIEPDQK.D (2+) K.QVVIMSTVCEHCGYR.S (3+) K.VESVVDLAR.D (2+) K.SESCALECPELK.L (2+) K.LSVNPGTLGGR.F (2+) R.FTTVEGLLTQVR.D (2+) R.DDLHQQIFDVGDAGEGGDGMESESK.K (3+) K.IFFDGIDEAIK.G (2+) K.IFFDGIDEAIKGER.K (3+) R.TAEEEEDLGLSDMK.T (2+) K.TEGYVDEVGDEK.R (2+)
8	XP_001395304	K.GTPLIYEYTYHEMK.I (3+) K.IIVDGEFDISDETGQK.V (2+) K.GDV FYFPK.G (2+)
9	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DGDYNGSSMTCYYVDK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) K.GDNPIWEDAK.V (2+) K.GDNPIWEDAKVPDEVR.K (3+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (3+)
10	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DGDYNGSSMTCYYVDK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) K.GDNPIWEDAK.V (2+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (2+)
11	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (3+) K.DANFDNQPSIHLLYMNK.D (3+) K.GDNPIWEDAK.V (2+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (2+)
12	XP_001592251	R.ESIEIAQNCISESFK.V (2+) K.DSQNLLQIYSVYEK.L (2+) R.QSQAAAAPPKPANETPTGK.Q (3+) K.ADAEAAVDADPK.Y (2+) R.VQELEAGGLDEDEVAER.G (2+)
13	XP_001587181	M.APSQLPAIFNPTSQDIEQLLAAQCHLGSK.N (3+) K.NLQVHMEPYLWK.T (3+) K.TRPDGINVINIGK.T (3+) R.IIAAIDNPADICVISARPYGQR.A (3+) K.FAAHTGAVAIAGR.F (2+) R.FTPGNFTNYITR.S (3+) R.LIIVTDPR.T (2+) R.DPEAEENKDAIEEAK.A (2+)

Table S5-1. Continued

14	XP_001591168	K.IHQVIGAVVDVK.F (2+) K.FDTEKLPPILNALETDNGGNK.L (3+) K.LPPILNALETDNGGNK.L (2+) K.LILEVAQHLGENVVR.T (3+) R.TIAMDGTEGLVR.G (2+) R.ATDTGSPIMVPVGAGTLGR.I (2+) R.IMNVTGDPIDER.G (3+) R.LPIHADAPPFTEQSTTAEVLVTGIK.V (3+) K.VVDLLAPYAR.G (2+) K.IGLFGGAGVGK.T (2+) K.TVFIQELINNIAK.A (3+) K.AHGGYSVFTGVGER.T (2+) R.EGNDLYHEMQETSVIQLDGDSK.V (3+) K.VALVFGQMNEPPGAR.A (2+) R.VALTGLTLAEQFR.E (2+) R.FTQAGSEVSALLGR.I (3+) R.IPSAVGYQPTLAVDMGSMQER.I (3+) R.GISELGIYPAVDPLDSK.S (2+) R.VVGQDHYDTATR.V (2+) R.VQQMLQEYK.S (2+)
15	XP_001585959	R.LYDTQPSTSLTSQDGIR.Y (2+) R.YLQSGYNAAR.K (3+) R.KWLVEGLGYK.D (2+) K.WLVEGLGYK.D (2+) K.WLVEGLGYKDVDINDK.A (3+) K.ADDKTGVFGYPNFFYSDGER.G (3+) K.TGVFGYPNFFYSDGER.G (2+) R.GGPVTTYLQSSLQR.S (2+) R.HGDTATGVTALVNGVETFISVTPTGR.V (3+) K.YSYESPPPEDR.D (2+) K.YSYESPPPEDRDLYLNSR.S (3+) R.DLYLNSR.S (2+) R.SGPYTFASETSVFWTTISHADGTK.A (3+) R.GQVNHWSSSCR.L (2+) K.AAELILK.S (2+)
16	XP_001585959	R.LYDTQPSTSLTSQDGIR.Y (3+) R.YLQSGYNAAR.K (2+) R.KWLVEGLGYK.D (3+) K.ADDKTGVFGYPNFFYSDGER.G (3+) K.TGVFGYPNFFYSDGER.G (2+) R.GGPVTTYLQSSLQR.S (2+) R.HGDTATGVTALVNGVETFISVTPTGR.V (3+) K.YSYESPPPEDR.D (3+) K.YSYESPPPEDRDLYLNSR.S (3+) R.DLYLNSR.S (2+) R.SGPYTFASETSVFWTTISHADGTK.A (3+) R.GQVNHWSSSCR.L (2+) K.AAELILK.S (2+)
17	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DGDYNGSSMTCYYVDK.D (2+) R.MKGDNPIWEDAK.V (3+) K.GDNPIWEDAK.V (2+) K.MGIVEIR.R (2+)
18	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.GDNPIWEDAK.V (2+) K.MGIVEIR.R (2+)
19	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (3+) K.DGDYNGSSMTCYYVDK.D (2+) K.GDNPIWEDAK.V (2+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (2+) K.WGDELPGTALACVIGNGK.I (2+)
20	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DGDYNGSSMTCYYVDK.D (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (2+) K.WGDELPGTALACVIGNGK.I (2+)
21	XP_001592529	K.AVKPIPYDDYTNR.L (2+) R.AWAFDSGEDR.L (2+) R.LLSYVER.A (2+) K.YIDIFAIYDLLNK.A (2+) K.ASTPSIAYGIAYVDDK.Y (2+) K.QYVSGYNSEVR.L (3+) R.ELDSQQAPNMDK.L (2+) K.LFHDYEFGEHFR.E (2+) R.ELVTSQSFINNEIR.T (2+) R.DTPAFSGTQFLDK.V (2+) K.WSYGLWENEGK.G (2+) K.EFYEAQIR.F (2+)
22	XP_001597509	K.TLGGRPLLEGKPLEIR.K (3+) R.TIAAQSAGPDSSVQTR.D (3+) R.DISADGIPVR.I (2+) K.MPVMLDDSLK.A (2+) K.AFEWAR.N (2+) K.DHIQGIVSLVPVTAHPSSIPAAYK.E (3+) K.SYEENAAGVPILDR.A (2+) R.AAMDVFLGAIEADPHDER.I (3+) K.HLDQFPPTYIATCGK.D (3+) K.DPLRDDGTVLEIMLK.E (3+) K.GRDEFLDNVCAGVK.F (2+)
23	XP_001587104	K.GPTIHVLYMTEDR.Q (3+) K.QGEILQGTSLACEITEK.N (3+) R.VFFQQHDGSICLYEYK.E (2+) K.EGDWHDQGIYIK.K (2+) K.KEEVQPPYQNPTPLACTMTK.N (3+) K.NGCIHLFFAGFDENNK.H (3+) K.KQDDVTDFFSGSK.L (2+) K.QDDVTDFFSGSK.L (2+) K.LGCTSDNNEVTLFYR.R (2+) R.KQPENDVGTMVYSDGK.W (3+) K.WKDGATVIPA.- (2+)
24	XP_001596229	R.VTIPVEEASAAAAAAK.E (2+) K.EVVDVVTPLTQSAYK.G (2+) K.IPISFFK.V (2+) R.VNMISPGYMDTALNNVPALDAQK.V (3+)
25	XP_001587104	R.SPDGGNVWVFQDSIIAQDAQISGSPMICYYVDK.D (3+) K.GPTIHVLYMTEDR.Q (2+) K.VKHLEGHTPIWTDVK.V (3+) K.HLEGHTPIWTDVK.V (2+) K.QGEILQGTSLACEITEK.N (3+) R.VFFQQHDGSICLYEYK.E (2+) K.EGDWHDQGIYIK.K (2+) K.EEVQPPYQNPTPLACTMTK.N (3+) K.NGCIHLFFAGFDENNK.H (2+) K.KQDDVTDFFSGSK.L (2+) K.QDDVTDFFSGSK.L (2+) K.LGCTSDNNEVTLFYR.R (3+) R.KQPENDVGTMVYSDGK.W (2+) K.QPENDVGTMVYSDGK.W (3+)

Table S5-1. Continued

26	XP_001596115	R.FAADPEDDDR.A (2+) R.SLGILTTDPLSTDLGK.E (2+) K.TDCVYFR.D (2+) R.NIVDMAAQIR.I (2+) R.IYATMAGGTEETSYTVLMLK.W (3+) K.WIGDFHDENNKPNPDQAK.L (2+) K.EGIQFVVDEELR.I (3+) K.QLQAGAQQALAGLENFHGQCEQHESGLK.V (3+) K.VNATSLEAQLIR.E (2+) R.AELDEYQAR.I (2+) K.YMWVWPVGTFIGVGLVQR.A (2+) K.IQDVMDEYEAK.Q (3+) R.TLELLQGAWK.S (2+) K.SMVTDLETIK.Q (2+) K.IVNEWNELR.E (2+)
27	XP_001596115	R.FAADPEDDDR.A (2+) R.AFIFNSSDMK.A (2+) R.SLGILTTDPLSTDLGK.E (3+) K.TDCVYFR.D (2+) R.NIVDMAAQIR.I (2+) R.IYATMAGGTEETSYTVLMLK.W (3+) K.EGIQFVVDEELR.I (3+) K.QLQAGAQQALAGLENFHGQCEQHESGLK.V (3+) K.VNATSLEAQLIR.E (2+) R.AELDEYQAR.I (2+) K.YMWVWPVGTFIGVGLVQR.A (3+) K.IQDVMDEYEAK.Q (3+) R.FVNGQVNNLTQEIRPAMR.T (3+) R.TLELLQGAWK.S (2+) K.SMVTDLETIK.Q (2+) K.QLINNDTDSIPPMILAKPQLQK.I (3+) K.IVNEWNELR.E (2+)
28	XP_001596115	R.SLGILTTDPLSTDLGK.E (2+) K.EGIQFVVDEELR.I (2+) K.VNATSLEAQLIR.E (2+) K.YMWVWPVGTFIGVGLVQR.A (3+) R.TLELLQGAWK.S (2+)
29	XP_001598610	K.TDAQGNATGSVEDSQIK.L (2+) K.LIGPLSVIGR.T (2+) R.TVVVHSGTDDLGR.G (3+) K.TGNAGTRPACGVIGIAA.- (2+)
30	XP_001588849	K.GTAQSVLGSVTGSTADK.E (2+) K.GTAQSVLGSVTGSTADKEAAAQK.K (3+) R.QEGSWNQTIGSGK.E (2+) K.EFIGGMLGAEELR.K (2+) R.GQQAQGQLNDLGSGVK.D (3+)
31	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DGDYNGSSMTCYYVDK.D (2+) K.GDNPIWEDAK.V (2+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (3+) K.WGDELPGTALACVIGNGK.I (2+)
32	XP_001598782	K.FSPYQLYPEASK.E (2+) K.VMTAYGGQSAGIDYK.F (3+) K.FNGTVANTLDAHR.V (3+) R.VIQHFQQEDVK.G (2+) K.IVMALYR.M (2+) R.MYFQEEK.H (2+) R.KIIEDEHEGLVDVK.N (2+) K.IIEDEHEGLVDVK.N (2+) R.EAEGNGVDSVPVVR.F (2+) R.DITLEGAQDVGEYVK.S (3+) K.SLEQIVK.E (2+)
33	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (3+) K.WGDELPGTALACVIGNGK.I (2+)
34	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (3+) K.DGDYNGSSMTCYYVDK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) K.GDNPIWEDAK.V (2+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (2+) K.WGDELPGTALACVIGNGK.I (2+) R.VFLQHHDYSIILYENQNNTWHDR.G (3+)
35	XP_001598122	K.TLNFITGNK.N (2+) K.GPVLVEDTCLCFNALK.E (3+)
36	XP_001587441	K.IVGYLNDAK.L (2+) K.LDPNTEVVIAPPALYLLLTR.E (2+) K.DSNITWTLLGHSER.R (2+) R.RVVLQEDDSFVASK.T (2+) R.VVLQEDDSFVASK.T (3+) K.TIEVVTAQLK.A (2+) K.IVIAYEPIWAIGTGK.V (2+) K.VASTEQAQEVHAAIR.S (3+) R.IIYGGSVSEK.N (2+) K.QEDIDGFLVGGASLKPAFVDIINSHL.- (3+)
37	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DGDYNGSSMTCYYVDK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) R.MKGDNPIWEDAK.V (3+) K.GDNPIWEDAK.V (2+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (2+) K.WGDELPGTALACVIGNGK.I (2+) K.VQATTPLACTMTK.D (2+) K.DGSVHLFYVSK.S (2+)
38	XP_001589786	R.WQNQFTWELAR.H (3+) R.HSIGEELVVYPAFTK.H (2+) K.GEEADDLPALESALESHESESMAR.S (3+)
39	XP_001592320	R.KSGVIVGDDVLK.L (3+) K.SGVIVGDDVLK.L (2+) K.SPIILQMSQGGAAYFAGK.G (3+) K.GVANGNQEASIAGGIAGAHYIR.A (3+) R.ALAPAYGIPVVLHTDHCAK.K (3+) K.LLPWLDGLLDADEK.Y (2+) K.EHGEPLFSSHMIDLSEEEVDYNIK.T (3+) K.LRPELLGK.H (3+) K.EDKPVFLVFHGGSGSSK.Q (2+) K.NKDYLMTPVGNPDGADKPNK.K (3+) K.DYLMTPVGNPDGADKPNK.K (3+) R.LAEGLKDFNTAGQL.- (2+) K.DFNTAGQL.- (2+)

Table S5-1. Continued

40	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (3+) K.DGDYNGSSMTCYYVDK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) R.MKGDNPIWEDAK.V (3+) K.GDNPIWEDAK.V (2+) K.GDNPIWEDAKVPDEVR.K (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (2+) K.WGDELPGTALACVIGNGK.I (2+) R.VFLQHHDYSIILYENQNNTWHDR.G (3+) K.DKVQATTPLACTMTK.D (3+) K.VQATTPLACTMTK.D (3+) K.DGSVHLFYVSK.S (2+) K.KEEELINFFPGSK.L (2+) K.EEELINFFPGSK.L (2+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (2+)
41	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (3+) K.DGDYNGSSMTCYYVDK.D (3+) R.MKGDNPIWEDAK.V (3+) K.GDNPIWEDAK.V (2+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (2+) K.DKVQATTPLACTMTK.D (3+) K.VQATTPLACTMTK.D (2+) K.DGSVHLFYVSK.S (2+) K.KEEELINFFPGSK.L (3+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (3+)
42	XP_001597411	M.PATTAETLSLVTR.N (2+) R.NVSVAPLVLLSAADHYGR.Q (2+) R.RVVGVLLGQNDGK.N (3+) R.VVGVLLGQNDGK.N (3+) K.LIGWYHTGPK.L (2+) R.ASDLEINELFK.R (2+) K.RYTPNPLLVIIDVQPK.E (3+) R.YTPNPLLVIIDVQPK.E (2+) K.EAGVPTDAYFAVEEIK.D (2+) K.TFVHTPSIIEAEEAEEIGVEHLLR.D (3+) R.DVAVGTLSTR.I (2+) R.ITNQVQSLQGLHAR.L (3+) K.ASSHIPNGLDAPVENNSELAHAMSIK.T (3+) K.TNDQLMAIYLSSLIR.A (2+) R.AITAFHDLIENK.I (2+)
43	XP_001595527	K.GFYENEVTQYATSSYEYVR.T (3+) R.NKQDIATILEYATK.R (2+) K.QDIATILEYATK.R (2+) K.EFTDFCAENR.I (2+) K.VHKDEDYEGSIGMK.C (2+) K.CLYPYSR.D (2+) K.MSDGLDSEGNEMVLAR.N (2+) R.NYDVCLNVVSSGNEFAALFPGK.A (3+) R.DFYNNHDDLPNIDPNLPQYPK.L (3+) K.LIYVWAQWVK.L (2+) K.LKDTDVFDEK.L (2+) K.LSDGCIDSISINHK.E (2+) K.IVAPENNGLFVCAQIQPFGGPESK.T (3+) K.NANNGTSYSWR.N (2+) R.NSTITCTLDCFYNFK.H (2+) K.ENEEGAIGENGVFSTK.D (3+) K.DMRPLWGSFGEYDFSK.I (3+) K.IWDAYHDSQEK.Y (2+) K.IWDAYHDSQEKYDK.L (3+) K.IHDPTGVFTPNTFCVPR.E (3+) R.EGEDTKDLLAER.V (2+)
44	XP_001598652	K.KGEPTPLADYK.G (2+) K.GEPTPLADYK.G (2+) K.VLLIVNTASK.C (2+) K.CGFTPQYEGLEK.L (3+) K.KVDVNGDNAAPLFK.W (3+) K.WLKEEKPGLLGLQR.V (3+) K.EEKPGLLGLQR.V (2+)
45	XP_001585797	K.RLESIIAPNLLVDYTTIGK.S (3+) R.LESIIAPNLLVDYTTIGK.S (2+) K.ISDDYVIGHHQLR.A (3+) K.FAGLKPLVR.W (2+) R.WNEGDGENDFLR.V (2+)
46	XP_001595639	K.YTVQSTGIWETIR.R (3+) R.VFAVDPTR.S (2+) R.SNGVPLNPQFR.N (2+) R.NPPPGSNEPFSFIDPVTLPAGDIAENPYWK.R (3+) R.RSYPQLSFVK.Q (3+) K.QGDVVGLLSVGSEASPR.K (2+) R.GLAAFFEGGDR.E (2+) K.GGLPPTPSGFSLR.E (2+) R.EGADKYSLTEENAYPEEYPCR.T (3+) K.YSLTEENAYPEEYPCR.T (2+)
47	XP_001591333	R.VGQFPGDENPACYVHGYVSTR.L (3+) K.TGQSDGIPMCIAATK.V (3+) K.VVNLVLALTPFNHSYDYR.S (3+) R.ATLLDPNENTEENIWAMK.L (2+) K.LITDGIVPER.W (2+) R.VPPDKSEITSTR.I (3+) K.NAGGGEAGNSFVSR.V (2+) R.VVGSMFGA.- (2+)
48	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (2+) K.WGDELPGTALACVIGNGK.I (3+) R.VFLQHHDYSIILYENQNNTWHDR.G (3+) K.VQATTPLACTMTK.D (2+) K.DGSVHLFYVSK.S (2+) K.KEEELINFFPGSK.L (2+) K.EEELINFFPGSK.L (2+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (2+)
49	XP_001595766	K.TIDFAGTK.E (2+) R.DNGLNVVVGVR.K (2+) K.NLFEVDEAISK.G (3+) K.GTIIMNLLSDAAQSETWPALKPQITK.G (3+) K.TLYFSHGFSPVFK.D (2+) K.VDVPTDVDVILVAPK.G (2+) R.GINSSIAVFQDVTGK.A (2+) K.AIALGVGVGSGYLYETTFEK.E (2+) K.EVYSDLYGER.G (2+) R.RGAIDWSGK.F (2+) R.GAIDWSGK.F (2+) K.FKDALKPVFNDLYDSVK.T (3+) K.DALKPVFNDLYDSVK.T (2+) R.DLEIWR.A (2+)

Table S5-1. Continued

50	XP_001595868	K.IYQASTTAPVNIAVVK.Y (2+) K.LNLPTNSSISVTLSQNDLR.T (2+) R.SALEEADSSLPK.L (2+) K.LASYPLK.I (2+) K.IISENNFPTAAGLASSAAGFAALVR.A (3+) R.AIANLYELK.S (2+) R.SLFGGYVAWEMGQK.E (2+) K.EDGSDSVAVEVAPASHWPTMR.A (3+) R.ALILVVSAEK.K (2+) K.DFEGFAK.V (2+) K.SVLEGVDGWNGK.E (2+) K.SVLEGVDGWNGKEVESK.S (3+) R.VILTGVGGGPEMTEK.H (2+)
51	XP_001588947	R.YSKPEEYGIVEVPLPK.L (2+) K.ACGVCGTDLHIHEGEFIAK.V (3+) R.VVADNSELCEECFYCR.R (3+) K.IENLSDVDATLLEPASCAAHGLEK.I (3+) K.KLDAADIYIELSR.D (2+) K.LDAADIYIELSR.D (2+) R.DNPEAQFQK.I (2+) K.YGFDIVVEATGSAK.I (2+) K.ILEDAINYVR.R (3+) K.LVVYGVYSSSAR.V (3+) K.VKVEGIVNK.T (2+) K.LEQWGEALESIK.N (3+)
52	XP_001584664	K.AIEIYFPSQCVAQEELEK.F (2+) K.AIEIYFPSQCVAQEELEKFDGVSAGK.Y (3+) K.YTIGLGQTK.M (2+) K.YNIDTNTIGR.L (2+) R.LEVGTETLLDK.S (2+) R.DAIVVAGDIALYAK.G (3+) K.CYTEAVDACYK.A (3+) R.EQTLKPLANGHINGAVSEDSTK.S (3+) R.FDYMTFHAPTCK.L (3+) R.MLYNDYLANPTASTFADVPAELR.D (3+) K.TFMGLTK.K (2+) R.IGVFSYGSGLAASLFSLK.I (3+) K.INGSTETMANALNLQER.L (2+) R.RTVAPEVYEEFCNLR.K (3+) R.TVAPEVYEEFCNLR.K (3+) K.SYTPQGSPDTIAK.D (3+) K.SVDDMFR.R (2+)
53	XP_001597967	R.GITISTAHIEYATEAR.H (2+) R.HYSHVDCPGHADYIK.N (3+) K.VDALEDPEMLELVEMEMR.D (3+) K.IDELMNAVDTWIPTPQR.D (3+) R.DLDKPFLMSVEDVFSIPGR.G (3+) K.DSEVEIVGK.G (2+) K.VTDIETFK.K (2+) K.VTDIETFKK.S (2+) R.AGDNSGLLLR.G (2+) R.GMIISAPGTTK.A (2+) K.FLVSMYVLTK.E (2+) R.TADEAAALHWPEGTEDADSK.M (3+) K.MVMPGDNVEMQCEIEKPCAMEVGQR.F (3+) R.TVATGLVTR.I (2+)
54	XP_001588993	K.QMYIDMVSMR.R (2+) R.GFCHLSTGQEAVAVGIEHSLTK.E (3+) K.EDDIITAYR.C (2+) K.SIIGELLGR.R (2+) K.GFYGGNGIVGAQVPVGAGLAFAHK.Y (3+) K.NVSVALYGDGASNQGQVFEAFNMAK.L (3+) K.LWNLPVLFGCENNK.Y (2+) R.SSALTDYYK.R (2+) R.GQYIPGLK.V (2+) K.VNGMDALAVK.A (2+) K.EYANAGNGPLVLEYVTYR.Y (3+) R.YGGHSMSDPGTTYR.T (3+) K.LIEWNVTTEDELK.T (3+) K.EAEEMPFPDATPQILYEDIYVR.G (3+) R.TNDENYYY.- (2+)
55	XP_001592632	R.ILPAASHQPFTPR.E (3+) R.WDPFDLDETVDWVHSLK.L (3+) K.LVAGAGDPTLK.H (2+) K.HGIGIFIFAAGK.N (3+) R.VELPEGPVR.G (2+) R.DFQAPVANFEENTNGQFK.I (3+) R.WLVGEDTFRPPWYHR.N (3+)
56	XP_001585148	K.ILDDISHR.R (2+) R.GTWLLVSPHR.T (3+) K.ITLPEYDPACYLCPGNK.R (2+) K.DTFVFVNDYSAVK.E (2+) K.EVQAEYAQDGESK.D (2+) K.DLSNILLR.A (2+) R.HLLEDYVK.L (2+) R.GLVDLNDEEK.L (2+) K.LGFAEAIAEVTR.R (2+) K.FLVGYELMAEPQR.D (2+) K.LRDCGGELYR.K (3+)
57	XP_001587768	K.RGTLGGTCLNVGCIPSK.A (3+) R.GTLGGTCLNVGCIPSK.A (2+) K.GVEFLFK.K (2+) K.VNLSDGGEETIVGK.N (3+) K.NILIATGSEATPFPGLEVDEK.R (3+) K.NILIATGSEATPFPGLEVDEKR.I (3+) K.VTVVEFLPQIGGPGMDAEIAK.S (3+) K.VTVVEFLPQIGGPGMDAEIAK.S (2+) K.GGKEETLDADVVLVAIGR.R (2+) K.EETLDADVVLVAIGR.R (2+) R.RPYTAGLGLENIGLETDDK.G (3+) R.RPYTAGLGLENIGLETDDKGR.L (3+) R.LVIDSEYR.T (2+) K.AEEEAVAVVEYIK.K (3+)
58	XP_001554714	K.DVDIAVAAAR.K (2+) K.NADLLAAVESLDNGK.A (2+) K.VIDTDPEYFTYTK.Q (2+) K.IGPAVATGNTVVLK.T (2+) K.EAGFPPGVVNIISGFGK.I (2+) K.VTLELGGK.S (2+) K.VGDPFHHETFQGPQVSQLQYDR.I (3+) K.GYFIQPTIFSDVTEDMK.I (2+) K.IMQEEIFGPVCSIAK.F (2+) K.FSTEEEIIK.I (2+) R.ELGEAALSNYTQTK.S (2+)

Table S5-1. Continued

59	XP_001597573	K.QVSIEAGSALIQACEK.A (2+) R.MCLVEVEK.A (2+) R.FANDIAGAPELGSTGR.G (3+) K.HSESIDVLDGLGSNIR.V (3+) R.LNDDVNEEWINDK.T (2+) K.FLPVSWEQALVEIGAAYK.D (3+) K.AIAGELIETESMVAMK.D (3+) K.IWGVEEADVILIVGSNTR.H (3+) K.RPMIIVGSGVTDHADAK.A (3+) K.HASNFLTEEWNGYNVLQR.A (3+) K.FIWLLGADEFNAADIPK.D (2+) R.GAQIADVVLPGAAYTEK.A (2+) R.AVSEFLGAPLPYDDVAALR.D (2+) R.MVEISPSLASYDVVEPVALK.Q (2+) K.VIDNFYFTDVISR.S (2+) K.ETGNPETNFLAPGYSSENPR.G (3+)
60	XP_001595527	K.EIAIQDLENLVNTLGFSNCFK.K (3+) R.TLPALILKPR.N (2+) R.NKQDIATILEYATK.R (2+) K.QDIATILEYATK.R (2+) K.EFTDFCAENR.I (2+) R.DLFNAILGGSPGNFGVVTHFTIK.V (3+) K.MSDGLDSEGNEMVLAR.N (2+) R.NYDVCLNVVSSGNEFAALFPGK.A (3+) R.DFYNNHDDLPNIDPNLPQYPK.L (3+) K.LKDTDVFDEK.L (2+) K.LSDGCIDSISINHK.E (2+) K.IVAPENNGLFVCAQIQPFGGPESK.T (3+) K.NANNGTSYSWR.N (2+) R.NSTITCTLDCFYNFK.H (2+) K.ENEEGAIGENGVFSTK.D (2+) K.IWDAYHDSQEK.Y (2+) R.EGEDTKDLLAER.V (3+)
61	XP_001595527	K.EIAIQDLENLVNTLGFSNCFK.K (2+) K.GFYENEVTQYATSSYEYVR.T (2+) R.TLPALILKPR.N (2+) R.NKQDIATILEYATK.R (2+) K.QDIATILEYATK.R (2+) R.LHTSVSWSLK.E (2+) K.EFTDFCAENR.I (2+) R.SFGLLADHVLEIDIVDYEGIPR.R (3+) R.DLFNAILGGSPGNFGVVTHFTIK.V (2+) K.MSDGLDSEGNEMVLAR.N (2+) R.NYDVCLNVVSSGNEFAALFPGK.A (3+) R.DFYNNHDDLPNIDPNLPQYPK.L (2+) K.LIYVWAQWVK.L (2+) K.LSDGCIDSISINHK.E (2+) K.ELMSYPMSYMTGSWWILDAVR.E (3+) R.EYPYPYIK.S (2+) K.IVAPENNGLFVCAQIQPFGGPESK.T (2+) R.NSTITCTLDCFYNFK.H (2+) K.IWDAYHDSQEK.Y (2+) R.KIHDPTGVFTPNTFCVPR.E (3+) K.IHDPTGVFTPNTFCVPR.E (3+)
62	XP_001586241	K.TPTTQTAAAMFQELCYDNK.D (3+) K.DQLSAGLIIAGWDER.H (2+) K.QSYAIGGSGSTYIYGYCDANWK.E (3+) K.EGMEEADAVDFVK.G (2+) K.GALQEAIK.W (2+) K.WDGSSGGVIR.M (2+) R.HLYLPDTNYAVR.H (2+)
63	XP_001595639	R.VFAVDPTR.S (2+) K.QGDVVGLLSVGSEASPR.K (2+) R.GLAAFFEGGDR.E (2+) K.AALEVFGK.G (2+)
64	XP_001598204	R.DAVIIDPANPEEVAPVLQPQLK.D (2+) K.LLDYPGFEGLPIIGGK.D (2+) K.NGEGFNLGVIK.V (2+) K.ALYTPCHTQDSICWFMEDDK.G (3+) K.AIFTGDTLFHGGCGR.F (3+) K.TLAAVPDDTVVYPGHEYTK.D (3+) K.FAVSVSQSVPVK.A (2+)
65	XP_001585237	K.TIVITGASSGIGR.S (2+) R.STAFEFAR.T (2+) K.LDVSKPEEVK.G (3+) K.GFVGGLPEEWR.E (2+) R.EIDILVNNAGLVK.G (2+) K.GDIINIGSIAGR.E (2+) R.VIEIDPGQVETEFSVVR.F (2+) R.ENVVIADTLVFPNHQAGAGGAMHR.K (3+)
66	XP_001591860	K.TTATISSFGGK.L (2+) K.KVPVIFYLAGLTCTGDNGAEK.G (3+) K.VPVIFYLAGLTCTGDNGAEK.G (3+) K.GFFQSAASEK.G (2+) K.GIAVVYPDTSPR.G (2+) R.GLNIAGENDSWDFGTGAGFYIDATNPPYSTNYK.M (3+) K.MYSYITSELPSSLFSSFPELDSSR.K (3+) K.SVSAFAPISNPVK.C (2+) K.GEFPALIDVGTGDNFYK.Q (2+)
67	XP_001586350	K.ETAGLITPK.F (2+) K.VYNEIAAR.L (2+) R.LEEKDDYDDGSYGPVLVR.L (3+) K.DDYDDGSYGPVLVR.L (3+) R.LAWHASGTFDK.A (2+) R.FAPEGDHGANAGLVAAR.D (3+) R.DFLQPVK.A (2+) R.MGFNDQEIVALSGAHALGR.C (3+) R.SGFEGPWTFSPTVVTNEYYK.L (2+) K.TLMMLPTDMALVSDK.T (2+) K.YAADESLFMK.D (2+) K.DFANVITK.L (2+) K.LFELGVPFAETTEQGPMK.F (2+)
68	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DGDYNGSSMTCYYVDK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) K.GDNPIWEDAK.V (2+) K.MGIVEIR.R (2+) K.DPWHTETILEEK.W (2+) K.WGDELPGTALACVIGNGK.I (2+) K.DKVQATTPLACTMTK.D (3+) K.VQATTPLACTMTK.D (2+) K.DGSVHLFYVSK.S (2+)

Table S5-1. Continued

69	XP_001594896	K.WVVLFSHPEDYTPVCTTELGAFAK.L (3+) K.LIGLSANTIESHGGWIK.D (2+) K.LTFPIIGDK.E (2+) K.VAYAYDMLDHQDTTNVDSK.G (3+) K.GIAFTIR.S (2+) R.SVFVIDPK.K (2+) R.LILSYPASTGR.N (2+) R.VVDSLQTGDK.H (2+) R.ITTPINWIPGDDVIVHPSVK.N (3+)
70	XP_001590668	K.AVEDHFDPTYK.Y (2+) K.YIGIGSGSTVVYVVEAIAAK.G (2+) R.EITSQMIFVPTGDQSK.Q (3+) R.ESLDVAFDGADEIDEDLNCIK.G (2+) K.GGGACLFQEK.L (2+) K.FVCVADYR.K (2+) K.AIPIEIAPLAAPTIK.R (2+) R.ILITLGSPDPK.I (2+) K.AGPVVTDNGMWIIDAPFPK.L (3+) R.IVGVLETGLFHGR.N (2+) R.NGAQVANAGEEGGGQKPVAAYFGMEDGNVEVR.T (3+)
71	XP_001588588	K.AAVAWDAGQELSIEDIEVAPPK.A (3+) R.IEIYYTGVCHTDAYTLSGK.D (3+) R.TCLLGCGITTGYGAAVETAK.V (2+) K.IIVVDVNPSK.K (2+) K.FGATDFVNPTELK.G (2+) R.SQLPQLVDDYMQGK.L (2+) K.LKVDEFITHR.Q (2+) K.VDEFITHR.Q (2+) R.QPLNGINQAFDDMK.K (2+)
72	XP_001591173	R.NAIEHDDIDIVAVNDPFIETEYAAYMLK.Y (3+) K.VLSDGLEVNGK.K (2+) R.DPANIPWAESEAYYVVESTGVFTTTEK.A (3+) K.VINDEFTIIEGLMTTIHSYTATQK.T (3+) R.VPTANVSVVDLTVR.I (2+) K.AASYDEIKEVIK.K (3+) K.GILAYTEDDVVSTDMNGDNHSSIFDAK.A (3+) K.LVSWYDNEWGYSR.R (2+) R.RVLDLLHYISK.V (2+) R.VLDLLHYISK.V (3+)
73	XP_001588622	K.IGERPQLDEIEK.A (2+) K.LITVTHVDTSTGVLSELQK.L (3+) K.AIGCPAGLSISYYSGR.A (3+) R.KTPPGSYFASFK.N (3+) K.NWLPIMQNYEAK.K (3+) K.QAVADLGLK.Q (2+) K.QLASNPADQASGMTAIYLPENVK.A (3+) K.AADLLPSLGK.K (2+) K.GVIFAGGLHK.E (2+) R.VGHMGVSVMDEGR.G (3+) K.AIEALGVGLK.E (2+)
74	XP_001556886	K.IADQVSDAILDACLAEDPLSK.V (2+) K.TGMVMVFGEITTK.A (2+) R.DGSLPWLRPDTK.T (2+) K.TQVTIEYK.H (2+) K.VHTVVVSAQHDENITTESLR.K (3+) R.KIIVDTYGGWGAHGGGAFSGK.D (3+) K.IIVDTYGGWGAHGGGAFSGK.D (3+) K.TSDELVEIIR.A (2+) R.ANFDLRPGVIVK.E (2+) K.ELDLAKPIYFK.T (2+) K.NGHFTSQEFSWEKPK.T (3+)
75	XP_001586239	K.FITDPYLR.L (2+) R.SPGDSSITIFRPNK.N (2+) K.CLILEEFDR.S (2+) K.VGGNYAPVLR.W (2+) R.NEGYGITLHLDSQTR.S (2+) R.SEIDEFSTSGFIGAK.K (2+) K.KDGDNITLVVPDSK.N (2+) K.DGDNITLVVPDSK.N (3+) K.NVIASVTSDSILEIGK.S (2+) K.TTEYIPSSSEDPGAICQK.L (3+) K.DTFGWNK.T (2+)
76	XP_001595378	K.AAVDEYFK.H (2+) R.VAEYATLTR.H (2+) R.FAYGEPFYQAIAR.H (3+) K.VLDVGCGVGGPAR.E (2+) K.FTGAHITGLNNNDYQIQR.A (3+) R.LGIELGDGISNMVK.I (2+) K.ISEGIAAIK.A (2+) K.AAGFELVLHEDLAK.R (2+) K.RPDATPWYYPLAGDFK.H (3+) K.HMGTIGDFFTIAR.M (3+) K.FVGVLEYLK.L (2+) K.TADSLAVAADCLVQGAK.E (2+) K.EDLFTPMYLMVAR.K (2+)
77	XP_001584655	K.EIYVAVIGAGGVGK.C (2+) K.CFLSQLEALSQR.Y (2+) K.ISLIFVSR.S (2+) K.YTPLSYSNLSTTLADSTQEPLSIPK.I (3+) K.IIEYLAAAPAK.V (2+) K.VVLVDNTSSQDVADAYPQVLSK.G (3+) K.GISIVTPNK.K (2+) K.LWQDVFSAASSSGAK.V (2+) K.VYHESSVGAGLPVISTLK.E (3+) K.ELVDTGDSVTK.I (2+) K.ELGYTEPDPR.D (2+) R.DDLNGLDVAR.K (2+) R.LAGLEVESPTSFPVQSLIPK.E (2+) K.ELESVSSGEEFLQK.L (3+) K.LPEFDSQMEETK.I (2+) K.GSDNIISFYTK.R (2+) R.YGSNPLIIQGAGAGGDVTAMGVTGDLIK.V (3+)
78	XP_001593497	K.VKNPIVELDGDEMTR.I (3+) K.NPIVELDGDEMTR.I (2+) K.FIHPYLDVDLK.Y (2+) K.YYDLGLEYR.D (2+) R.DETNDQVTIDAAEAIQK.Y (2+) K.CATITPDEAR.V (2+) R.NHLGGTVFR.E (2+) R.EPIVIPR.I (2+) K.SGGGVAQTQYNTDESISGFAHASFK.L (3+) K.GLPLYMSTK.N (2+) R.FKDIFQEIFDK.E (2+) K.DIFQEIFDK.E (2+) R.LIDDMVAQMMK.S (2+) K.SSGGYVMALK.N (2+) K.TFESEAAHGTVTR.H (2+) K.GNPTSTNPIASIFAWTR.G (2+) K.DLALACGNTGK.D (2+) K.DDYVTTGEYLEAVEK.R (2+)
79	XP_001597603	K.SYHITLVTPNTSFYFK.I (3+) R.FVTVSNEQGDTR.Q (2+) R.QIHYDSLIISTGTTSK.S (3+) K.SPLWGLHGNESITK.K (3+) K.VTLLSGANR.L (2+) R.AQDYLENMHVEVIHNVR.V (3+) R.EVDIYIDATGGSANSQFLPK.T (3+) K.KPATPAPGYLASLLK.M (3+) K.MFLGGSDGYPVQLEFKPLK.E (3+) K.SYFIELIGPIISGEK.W (2+)

Table S5-1. Continued

80	XP_001585697	K.NAHPISLDVTDDK.A (3+) K.NDLVISLIPYTFHATVIK.S (2+) K.NVVTTSYVSPAMMELDEEAK.N (3+) K.NAGITVMNEIGLDPGIDHLYAVK.T (3+) K.ITSFLSYCGGLPAPEDSDNPLGYK.F (3+) R.VLVDMGFLSDEDK.G (2+) R.VLVDMGFLSDEDKGFSEPISWK.E (3+) K.GFSEPISWK.E (2+) K.VLAASSSFEDDLK.W (2+) R.IMNGLNWVGIFSDEK.I (2+) R.GNPLDTLCATLEK.K (2+) R.TSTLVEYGDPK.G (2+) K.LVGVPCGVAVK.Q (2+) K.YGITLVEK.T (2+)
81	XP_001593815	R.TESDAFGNVEVPSDK.Y (2+) R.AIEILGGTMGSK.K (2+) R.THLQDATPLTLAQEFSGYVAQLDYGIER.V (3+) R.LLAQGGTAVGTGINTFK.G (2+) K.GFAEAIAEEVTK.M (2+) K.FEALAAHDAIVQASGSLTTLASSLFK.I (3+) R.CGLGELALPENEPGSSIMPGK.V (3+) K.NLVVGLQANEEK.I (2+) K.ESLMLVTCLNPK.I (2+) K.ALSEEDFDK.L (2+) K.LVRPELMVGPEDYK.K (3+)
82	XP_001585697	K.NAHPISLDVTDDK.A (3+) K.ITSFLSYCGGLPAPEDSDNPLGYK.F (3+) R.YNIPEAQTIIR.G (2+) R.YQGFPEFIR.V (2+) R.VLVDMGFLSDEDK.G (2+) R.VLVDMGFLSDEDKGFSEPISWK.E (3+) R.GNPLDTLCATLEK.K (2+) K.LVGVPCGVAVK.Q (2+)
83	XP_001594974	K.IQDVQDTPYFVDNK.F (2+) K.SESTQFIDLHDPATNNLVTR.V (3+) R.LAASITLEQGK.T (2+) R.GLQVAETACAIPQQMMGEVIEVAK.D (3+) R.DPGAAMILAELAQK.A (3+) K.AGFPNGVLNIVHGASK.T (3+) K.TVDFIIDEPAIK.A (2+) K.AISFVGSNR.A (2+) R.AGEYIFTR.G (2+) K.QHALNSIVGAAFGAAGQR.C (3+) R.CMALSTLVMVGETK.E (2+) K.EWLSELAESAK.A (2+) K.GADLGPVISPQSK.K (2+) K.RIEDLIASAEEEGATILLDGR.G (3+) R.IEDLIASAEEEGATILLDGR.G (2+) K.NIEAGQQGINVPIPVPLPFFSFTGNK.K (3+) K.NIEAGQQGINVPIPVPLPFFSFTGNKK.S (3+) K.TVTSLWR.S (2+)
84	XP_001554714	K.TFEVINPTTEEVITSVHEATEK.D (2+) K.DVDIAVAAAR.K (2+) K.NADLLAAVESLDNGK.A (2+) K.VIDTDPEYFTYTK.Q (3+) K.IGPAVATGNTVVLK.T (2+) K.EAGFPPGVVNIISGFGK.I (3+) K.VGDPFHHETFQGPQVSQLQYDR.I (3+) R.IMGYIDEGKK.S (3+) K.GYFIQPTIFSDVTEDMK.I (2+) K.IMQEEIFGPVCSIAK.F (2+) K.FSTEEEIIK.I (2+) K.IGNGSNYGLASAVHTQNLNTALR.V (3+) R.ELGEAALSNYTQTK.S (2+)
85	XP_001587807	K.NNFINYK.K (2+) K.MSENLSIVR.Q (2+) R.LNRPLAYAEK.I (3+) K.EVYDFLSTSCAK.Y (2+) K.LTGELSGWATPK.D (2+) K.VAGILTVK.G (2+) R.MYDYLAATK.R (2+) R.TEIGDFAR.T (2+) K.ANNWPEELK.V (2+) K.VGLIGSCTNSSYEDMTR.A (2+) K.SLFTVTPGSEQIR.A (3+) K.KGEANSIISSYNR.N (2+) R.GYDPGQDTYQAPPAER.A (2+) R.ASVNVAVSPTSDR.L (2+) R.LQLLEPFKPWDGK.D (2+) K.DALDMPILIK.A (2+) K.TTTDHISMAGPWLK.Y (2+) K.NFTTGEFDAVPATAR.D (2+) K.KGIPWVVVGDWNYGEGSSR.E (3+) K.GIPWVVVGDWNYGEGSSR.E (2+) R.HLGGLAIITK.S (2+) K.QGMLPLTFINPEDYDKIDPYDK.V (3+) K.LAHTFNAPQIEWFK.N (3+)
86	XP_001593324	R.GKATLPDLPYDYGALEPSISGK.I (3+) K.ATLPDLPYDYGALEPSISGK.I (3+) K.HHQTYVNSFNTASEQLEAAESK.G (3+) K.TAIETSYGDFSSFK.T (3+) K.FNAALAGIQGSGWAWLVK.D (3+) K.TYANQDPVVGK.F (3+) K.FTPLLGVDAWEHAYYLQYENR.K (3+) R.KAEYFSAIWDVINWK.T (3+) K.AEYFSAIWDVINWK.T (2+)
87	XP_001584968	K.DGDYNGSSMTCYYVDK.D (3+) K.DANFDNQPSIHLLYMNK.D (2+) K.GDNPIWEDAK.V (2+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.DPWHTETILEEK.W (2+) K.WGDELPGTALACVIGNGK.I (2+) R.VFLQHHDYSIILYENQNNTWHDR.G (3+) K.DKVQATTPLACTMTK.D (2+) K.VQATTPLACTMTK.D (3+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (2+) K.EEELINFFPGSK.L (2+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (2+)
88	XP_001596374	K.VSDAVTDVTAALGNTSISDKPAETTTGEK.A (3+) K.AANTTTDKEAVLASAAEGR.R (3+) K.EAVLASAAEGR.R (2+) R.LYIGNLAYATTEGELQAFFK.G (3+) K.GYLIESTSIPK.N (2+) K.VSVQLAR.K (2+) R.TDESAAEAPAAASEVLPLTETTNQATTETK.E (3+) R.ERGPPADGIPSK.N (3+) K.VMVANLPYDLHEDK.L (3+) K.VMVANLPYDLHEDKLK.E (3+) K.ELFAIYEPTSAK.I (2+) K.IALRPIPR.F (3+) R.GFGFVTLSSEEQQQK.A (3+) K.TDDDVNANAANATAETAAAPPAGAETVSA.- (3+)

[a] Spot number as given on the 2-D gel image.

[b] Accession number in NCBI database.

[c] Sequence of matched peptides by LC-MS/MS.

Table S6-1. Detailed mass spectrometric data of proteins identified by 1-DE with LC-MS/MS in the sclerotial exudates

Band[a]	Accession no.[b]	MS/MS Peptide sequence (charge)[c]
1	XP_001591237	K.LYLQSGYNIVAGGLK.N (2+) R.VIVSAGAFGTPK.L (2+) R.IGPLAQAAPDIGPMFWEQITPGDGIPR.A (3+)
1	XP_001591237	K.LYLQSGYNIVAGGLK.N (3+) K.LYLQSGYNIVAGGLK.N (2+) R.IGPLAQAAPDIGPMFWEQITPGDGIPR.A (3+) R.VEGDTPNAMTLSSYLGR.G (2+) K.YDLDAVATAIDHMVAALSTVK.N (3+)
2	XP_001591716	R.DSVPAGYVAAPYYPTPK.G (3+) K.AQTVVAQMTLAEK.V (2+) K.GVNIILGPTVAPTGR.K (2+) R.TLHELYMWPFAEGIK.A (3+) R.IVATWYQLGQDK.S (2+) K.SVVQLYVQYPSDSPYDTPIIQLR.D (3+)
2	XP_001591716	R.TLHELYMWPFAEGIK.A (3+) K.SVVQLYVQYPSDSPYDTPIIQLR.D (3+)
3	XP_001594393	K.YGTVAQLPLVGTITNPLSGITVGR.A (3+) R.AQTSDNVVVELAATSHAGLYQYTFPTTSTANK.I (3+) K.ILIDVSHVLPSFR.G (3+) K.SVWNNQVLSR.I (2+) R.SLIDIWR.H (2+) R.TQGGSNADNVLADAYVK.G (3+) K.YGYITPTYSR.A (2+) R.AVDYAYNDFSLYQVASGLGK.T (2+)
3	XP_001594393	K.YGTVAQLPLVGTITNPLSGITVGR.A (3+) K.ACQNVNNEIPHDTIFSTVVSDTK.S (3+) K.WTSTEPYYQDIFTLWDLFR.C (3+) R.CSTALFHVLQPTAYEEYIR.S (3+) R.GQINWNDGYAAMVK.D (2+) K.LTITSTGGDGNGDNDIYVQSLK.V (3+)
4	XP_001594393	K.YGTVAQLPLVGTITNPLSGITVGR.A (2+) K.YGTVAQLPLVGTITNPLSGITVGR.A (3+) R.AVPDQGSVGYYR.A (2+) R.AQTSDNVVVELAATSHAGLYQYTFPTTSTANK.I (3+) K.ILIDVSHVLPSFR.G (2+) K.ILIDVSHVLPSFR.G (3+) R.VGISWISK.E (2+) K.SVWNNQVLSR.I (2+) R.CSTALFHVLQPTAYEEYIR.S (3+) R.SLIDIWR.H (2+) R.TQGGSNADNVLADAYVK.G (3+) R.GQINWNDGYAAMVK.D (2+) R.GQINWNDGYAAMVK.D (3+) K.YGYITPTYSR.A (2+) R.AVDYAYNDFSLYQVASGLGK.T (2+) K.LTITSTGGDGNGDNDIYVQSLK.V (2+) K.VNGVLWDK.N (2+)
4	XP_001594393	K.YGTVAQLPLVGTITNPLSGITVGR.A (2+) K.YGTVAQLPLVGTITNPLSGITVGR.A (3+) K.ILIDVSHVLPSFR.G (3+) K.ACQNVNNEIPHDTIFSTVVSDTK.S (3+) K.WTSTEPYYQDIFTLWDLFR.C (3+) R.CSTALFHVLQPTAYEEYIR.S (3+) R.SLIDIWR.H (2+) R.TQGGSNADNVLADAYVK.G (3+) R.GQINWNDGYAAMVK.D (2+) R.GQINWNDGYAAMVK.D (3+) R.AVDYAYNDFSLYQVASGLGK.T (2+) K.LTITSTGGDGNGDNDIYVQSLK.V (3+)
4	XP_001561160	R.AVPDQGSVGYYR.A (2+) R.VGISWISK.E (2+) R.SLIDIWR.F (2+) R.TQGGSNADNVLADAYVK.G (3+) R.GQINWDDGYAAMVK.D (2+) R.GQINWDDGYAAMVK.D (3+) K.VNGVDWDK.N (2+)
4	XP_001561160	R.SLIDIWR.F (2+) R.TQGGSNADNVLADAYVK.G (3+) R.GQINWDDGYAAMVK.D (2+) R.GQINWDDGYAAMVK.D (3+)
4	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.APTLEALLYDPTK.A (2+) R.FSTLATSTIPR.L (2+) R.MLHLDNTGFVAGATQQK.L (3+)
4	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.NIDLEILVK.N (2+) R.IQPLSANPTWELDSMPEGR.G (3+) K.LKPDAADEFVTEFR.V (3+)
4	XP_001591947	R.QQYFTQLVGDAITLPTIK.G (3+) K.GDGVTDDTAAIQAIFDK.A (3+)
4	XP_001591947	K.DFGAVGDGVTDDTAAINAAISAGNR.C (3+) K.GCDSTTLTPALVYFPPGTYAISTPIR.Q (3+) R.AAGSATIMDSTITNTPIGISTVYDIK.E (3+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) K.GDGVTDDTAAIQAIFDK.A (2+) K.GDGVTDDTAAIQAIFDK.A (3+) K.GDGVTDDTAAIQAIFDK.A (3+) K.ATTDQIVYFDHGGYVVTDTIK.V (3+) R.NTFAATIAMFK.E (2+)
5	XP_001594393	K.YGTVAQLPLVGTITNPLSGITVGR.A (3+) .AVPDQGSVGYYR.A (2+) R.VGISWISK.E (2+) K.SVWNNQVLSR.I (2+) R.SLIDIWR.H (2+) R.TQGGSNADNVLADAYVK.G (3+) K.YGYITPTYSR.A (2+) R.AVDYAYNDFSLYQVASGLGK.T (2+) K.LTITSTGGDGNGDNDIYVQSLK.V (3+)
5	XP_001594393	K.YGTVAQLPLVGTITNPLSGITVGR.A (3+) R.AQTSDNVVVELAATSHAGLYQYTFPTTSTANK.I (3+) K.ILIDVSHVLPSFR.G (3+) K.ACQNVNNEIPHDTIFSTVVSDTK.S (3+) K.WTSTEPYYQDIFTLWDLFR.C (3+) R.CSTALFHVLQPTAYEEYIR.S (3+) R.AVDYAYNDFSLYQVASGLGK.T (2+)
5	XP_001591947	R.QGAPAYGDASYQIFR.N (2+) R.QGAPAYGDASYQIFR.N (3+) K.GCDSTTLTPALVYFPPGTYAISTPIR.Q (3+) R.QQYFTQLVGDAITLPTIK.G (2+) R.QQYFTQLVGDAITLPTIK.G (3+) K.YGAFLGSQQFTSR.N (2+) R.AAGSATIMDSTITNTPIGISTVYDIK.E (3+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) R.TKPQYETLPASSFLSVK.S (3+) K.GDGVTDDTAAIQAIFDK.A (2+) K.GDGVTDDTAAIQAIFDK.A (3+)
5	XP_001591947	R.QGAPAYGDASYQIFR.N (3+) K.DFGAVGDGVTDDTAAINAAISAGNR.C (3+) K.GCDSTTLTPALVYFPPGTYAISTPIR.Q (3+) R.QQYFTQLVGDAITLPTIK.G (2+) R.QQYFTQLVGDAITLPTIK.G (3+) R.NTFAATIAMFK.E (2+) K.GLANFAGMALLDANPYDPQYNWFTNQNNFYR.Q (3+) K.INNCGIGLDMSAGGAASR.A (3+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) R.TKPQYETLPASSFLSVK.S (3+) K.GDGVTDDTAAIQAIFDK.A (2+) K.GDGVTDDTAAIQAIFDK.A (3+) K.ATTDQIVYFDHGGYVVTDTIK.V (3+) K.ITGEIWPMIMAK.G (2+)

Table S6-1. Continued.

5	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.NIDLEILVK.N (2+) K.ELPELAGDYR.T (2+) R.FSTLATSTIPR.L (2+) K.LKPDAADEFVTEFR.V (3+) R.VENYVPPYLSGDNANK.R (3+) R.MLHLDNTGFVAGATQQK.L (3+)
5	XP_001596362	K.NIDLEILVK.N (2+) K.NQPYYMYPFVHLLNDGNLFIFVSK.S (3+) R.IQPLSANPTWELDSMPEGR.G (3+) R.GMVEGNLLPDGTVIWLNGGNLGAQGFGLAK.A (3+) K.APTLEALLYDPTK.A (2+) K.LKPDAADEFVTEFR.V (3+) R.MLHLDNTGFVAGATQQK.L (3+)
5	XP_001554182	K.YGAFLGSQQFTSR.N (2+) R.TKPQYETLPASSFLSVK.S (3+) K.GDGTTDDTAAIQAIFDK.A (2+) K.GDGTTDDTAAIQAIFDK.A (3+)
5	XP_001554182	R.TKPQYETLPASSFLSVK.S (3+) K.GDGTTDDTAAIQAIFDK.A (2+) K.GDGTTDDTAAIQAIFDK.A (3+) K.ITGEIWPMIMAK.G (2+) R.NTFAATIAMFK.G (2+)
6	XP_001591947	R.QGAPAYGDASYQIFR.N (3+) R.QGAPAYGDASYQIFR.N (2+) K.DFGAVGDGVTDDTAAINAAISAGNR.C (3+) K.DFGAVGDGVTDDTAAINAAISAGNR.C (2+) K.GCDSTTLTPALVYFPPGTYAISTPIR.Q (3+) R.QQYFTQLVGDAITLPTIK.G (2+) R.QQYFTQLVGDAITLPTIK.G (3+) K.YGAFLGSQQFTSR.N (2+) K.YGAFLGSQQFTSR.N (3+) K.INNCGIGLDMSAGGAASR.A (2+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) R.TKPQYETLPASSFLSVK.S (3+) R.TKPQYETLPASSFLSVK.S (2+) K.GDGVTDDTAAIQAIFDK.A (2+) K.ATTDQIVYFDHGGYVVTDTIK.V (3+) K.ATTDQIVYFDHGGYVVTDTIK.V (2+) K.ITGEIWPMIMAK.G (2+) K.EASQGSVGMWDVHTR.I (2+) K.EASQGSVGMWDVHTR.I (3+) R.NTFAATIAMFK.E (2+)
6	XP_001591947	R.QGAPAYGDASYQIFR.N (2+) R.QGAPAYGDASYQIFR.N (3+) K.DFGAVGDGVTDDTAAINAAISAGNR.C (2+) K.DFGAVGDGVTDDTAAINAAISAGNR.C (3+) K.GCDSTTLTPALVYFPPGTYAISTPIR.Q (3+) R.QQYFTQLVGDAITLPTIK.G (2+) R.QQYFTQLVGDAITLPTIK.G (3+) K.GLANFAGMALLDANPYDPQYNWFTNQNNFYR.Q (3+) K.QQGIFMDNGSGGFMADLTFNGGK.Y (3+) K.INNCGIGLDMSAGGAASR.A (2+) K.INNCGIGLDMSAGGAASR.A (3+) R.AAGSATIMDSTITNTPIGISTVYDIK.E (3+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) R.TKPQYETLPASSFLSVK.S (3+) R.TKPQYETLPASSFLSVK.S (2+) K.GDGVTDDTAAIQAIFDK.A (3+) K.GDGVTDDTAAIQAIFDK.A (2+) K.ATTDQIVYFDHGGYVVTDTIK.V (2+) K.ATTDQIVYFDHGGYVVTDTIK.V (3+) K.ITGEIWPMIMAK.G (2+) R.NTFAATIAMFK.E (2+)
6	XP_001594733	R.LEYISDVSDFSR.S (2+) R.IFLDALPEDAPYTVPYGTVAIDYLSLK.F (3+) R.NAFSLTLDGK.L (2+) R.NVLADLADHQR.A (3+) K.CLDVFYPSVTDAGTTLIWK.G (3+) K.GVGVSGDYGDYAFLPR.I (2+) R.IGPVTYYSALYVLALENAAAIAK.S (3+) R.STSILNYLNSANR.R (2+)
6	XP_001594733	R.LEYISDVSDFSR.S (2+) K.CLDVFYPSVTDAGTTLIWK.G (3+) K.GVGVSGDYGDYAFLPR.I (2+)
6	XP_001594397	R.AFIVGDVNNPSNR.F (2+) R.FIYLVLDTASGDTAIR.Y (3+) R.FIYLVLDTASGDTAIR.Y (2+) K.QSYQAIVDGAVLSIK.R (2+) R.SLYAYLANPAAER.A (2+) K.GVAAYLFEK.A (2+) R.QYDAAQALYNSLDSVGTPLVDGSVK.S (3+)
6	XP_001594397	K.ADITGPVVEINFMGYASLPQVGTGLR.Q (3+) R.FIYLVLDTASGDTAIR.Y (2+) R.GPAFQALDLGVTSCYIIGQR.Q (3+) R.QYDAAQALYNSLDSVGTPLVDGSVK.S (3+) R.VGQLIIIISPSEATTMSGR.R (3+)
6	XP_001554182	K.YGAFLGSQQFTSR.N (2+) K.YGAFLGSQQFTSR.N (3+) R.TKPQYETLPASSFLSVK.S (3+) R.TKPQYETLPASSFLSVK.S (2+) K.GDGTTDDTAAIQAIFDK.A (2+) K.ITGEIWPMIMAK.G (2+) K.ETSQGSAGMWDVHTR.I (2+) R.NTFAATIAMFK.G (2+)
6	XP_001554182	R.TKPQYETLPASSFLSVK.S (3+) R.TKPQYETLPASSFLSVK.S (2+) K.GDGTTDDTAAIQAIFDK.A (3+) K.GDGTTDDTAAIQAIFDK.A (2+) K.ITGEIWPMIMAK.G (2+) R.NTFAATIAMFK.G (2+)
6	XP_001590125	K.FAVDSLAAGGQDYLTITSLSAR.Q (3+) R.QAFGAVQLCGTPDKPYYFLK.E (3+) K.WETLAIVPATASVPAHTK.L (3+) K.LAYQDSTGHGLLYNLYADTLLK.L (3+)
6	XP_001590125	R.QSVIGTYLYVSVK.S (2+) K.FAVDSLAAGGQDYLTITSLSAR.Q (3+) R.QAFGAVQLCGTPDKPYYFLK.E (3+) K.EISSDGNTQTVDVIFPAMPIFLYSNPVLLK.Y (3+) K.WETLAIVPATASVPAHTK.L (3+) K.LAYQDSTGHGLLYNLYADTLLK.L (3+) K.SDWEMWAAAIASPSTK.S (2+) K.SDWEMWAAAIASPSTK.S (3+)
6	XP_001596362	K.APTLEALLYDPTK.A (2+) R.FSTLATSTIPR.L (2+)
6	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.NIDLEILVK.N (2+) R.IQPLSANPTWELDSMPEGR.G (3+) R.GMVEGNLLPDGTVIWLNGGNLGAQGFGLAK.A (3+) K.APTLEALLYDPTK.A (2+) K.LKPDAADEFVTEFR.V (3+)
6	XP_001597035	K.YYTQSKPQYNTLSVSSFTSAR.T (3+) R.GINNAASSGQVFFLDAGTYK.V (3+)
6	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) R.GINNAASSGQVFFLDAGTYK.V (3+) R.NGQSQALYNANLNVYPTLITLYK.S (3+)

Table S6-1. Continued.

7	XP_001591947	R.QGAPAYGDASYQIFR.N (3+) R.QGAPAYGDASYQIFR.N (2+) K.DFGAVGDGVTDDTAAINAAISAGNR.C (3+) K.DFGAVGDGVTDDTAAINAAISAGNR.C (2+) K.GCDSTTLTPALVYFPPGTYAISTPIR.Q (3+) R.QQYFTQLVGDAITLPTIK.G (2+) K.YGAFLGSQQFTSR.N (2+) K.YGAFLGSQQFTSR.N (3+) K.INNCGIGLDMSAGGAASR.A (2+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) R.TKPQYETLPASSFLSVK.S (2+) K.GDGVTDDTAAIQAIFDK.A (2+) K.ATTDQIVYFDHGGYVVTDTIK.V (3+) K.ATTDQIVYFDHGGYVVTDTIK.V (2+) K.ITGEIWPMIMAK.G (2+) K.EASQGSVGMWDVHTR.I (2+) K.EASQGSVGMWDVHTR.I (3+) R.NTFAATIAMFK.E (2+)
7	XP_001591947	K.DFGAVGDGVTDDTAAINAAISAGNR.C (3+) K.GCDSTTLTPALVYFPPGTYAISTPIR.Q (3+) R.QQYFTQLVGDAITLPTIK.G (2+) R.QQYFTQLVGDAITLPTIK.G (3+) K.GLANFAGMALLDANPYDPQYNWFTNQNNFYR.Q (3+) R.AAGSATIMDSTITNTPIGISTVYDIK.E (3+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) R.TKPQYETLPASSFLSVK.S (3+) K.GDGVTDDTAAIQAIFDK.A (2+) K.ATTDQIVYFDHGGYVVTDTIK.V (3+) R.NTFAATIAMFK.E (2+)
7	XP_001590125	R.STFLMTVAGK.V (2+) R.QNQSEFNADYADDQAHWGYWLWSTK.A (3+) K.AVNGMSFQSGQDVVVR.G (2+) K.AVNGMSFQSGQDVVVR.G (3+) K.FAVDSLAAGGQDYLTITSLSAR.Q (3+) K.EISSDGNTQTVDVIFPAMPIFLYSNPVLLK.Y (3+) K.GIIAIEAMSR.I (2+) K.WETLAIVPATASVPAHTK.L (2+) K.WETLAIVPATASVPAHTK.L (3+) K.LAYQDSTGHGLLYNLYADTLLK.L (3+) R.IYDMQSEYYPTIANAYGVQLDSR.N (3+) K.SDWEMWAAAIASPSTK.S (2+) K.SDWEMWAAAIASPSTK.S (3+) K.LATWIGVTPTDR.A (2+)
7	XP_001590125	R.QSVIGTYLYVSVK.S (2+) R.QNQSEFNADYADDQAHWGYWLWSTK.A (3+) K.FAVDSLAAGGQDYLTITSLSAR.Q (3+) R.QAFGAVQLCGTPDKPYYFLK.E (3+) K.EISSDGNTQTVDVIFPAMPIFLYSNPVLLK.Y (3+) K.SDWEMWAAAIASPSTK.S (2+) K.SDWEMWAAAIASPSTK.S (3+)
7	XP_001588171	R.NLLCNIGSSGSCAAGASSGIVIASPDK.V (3+) K.VNPDYFYTWTR.D (2+) R.SIYSINSGIAEGVAVSVGR.Y (3+) R.DFSSSVTAGTYSSSTSTYTTLYNAIK.T (3+) R.AGGAPLSAYDLTWSYAAFLTAAAR.R (3+) K.IAGSISQLGSWAPASAVALSASK.Y (3+) K.FINVASDGTVTWEADPNR.S (3+)
7	XP_001588171	R.NLLCNIGSSGSCAAGASSGIVIASPDK.V (3+) K.DANSILSTIQTFDPTASCDSSTFQPCSDR.A (3+) R.SIYSINSGIAEGVAVSVGR.Y (3+) R.AGGAPLSAYDLTWSYAAFLTAAAR.R (3+) K.YTSSNPLWSVTINLPAGTAIQYK.F (3+) K.FINVASDGTVTWEADPNR.S (3+) K.FINVASDGTVTWEADPNR.S (2+)
7	XP_001594733	R.LEYISDVSDFSR.S (2+) R.IFLDALPEDAPYTVPYGTVAIDYLSLK.F (3+) K.CLDVFYPSVTDAGTTLIWK.G (3+) K.GVGVSGDYGDYAFLPR.I (2+) K.TNLVSSAFDEIR.R (2+)
7	XP_001594733	K.CLDVFYPSVTDAGTTLIWK.G (3+) R.IGPVTYYSALYVLALENAAAIAK.S (3+) R.FYGNSFYDNDVLSDGFSQR.V (3+)
7	XP_001554182	K.YGAFLGSQQFTSR.N (2+) K.YGAFLGSQQFTSR.N (3+) R.TKPQYETLPASSFLSVK.S (2+) K.GDGTTDDTAAIQAIFDK.A (3+) K.ATTDQVVYFDHGAYVVTDTIK.V (3+) K.ITGEIWPMIMAK.G (2+) K.ETSQGSAGMWDVHTR.I (2+) R.NTFAATIAMFK.G (2+)
7	XP_001554182	R.TKPQYETLPASSFLSVK.S (3+) K.GDGTTDDTAAIQAIFDK.A (2+) K.GDGTTDDTAAIQAIFDK.A (3+) R.NTFAATIAMFK.G (2+)
7	XP_001595513	K.INYVEVGNEDNLSDGASSYHAYR.W (3+) K.ATVPFTVTNSENSK.S (2+)
7	XP_001595513	K.LGISNPGWWGISVQPQK.Y (3+) K.INYVEVGNEDNLSDGASSYHAYR.W (3+) K.MDYGTWIGAVGEAIYLLGMER.N (3+) K.GTLTVLNAPDGNSMNVYGGEDVVVK.K (3+)
7	XP_001594397	R.AFIVGDVNNPSNR.F (2+) R.SLYAYLANPAAER.A (2+)
7	XP_001594397	R.FIYLVLDTASGDTAIR.Y (2+) R.FIYLVLDTASGDTAIR.Y (3+) R.QYDAAQALYNSLDSVGTPLVDGSVK.S (3+)
7	XP_001550527	R.STFLMTVAGK.V (2+) R.QDQSEFNADYADDQAHWGYWLWSTK.A (3+)
7	XP_001550527	R.QDQSEFNADYADDQAHWGYWLWSTK.A (3+) R.QAFGAVQLCGTPDKPYYFLK.E (3+)
8	XP_001597035	R.NLVFDMTGIPAGTTATGLHWPTSQATSLQNVEFR.M (3+) R.KYDPSGPSDFQGAITPNNRPASLLSGNK.Y (3+) K.YYTQSKPQYNTLSVSSFTSAR.T (2+) K.YYTQSKPQYNTLSVSSFTSAR.T (3+) R.TNGAIGDGVTDDTAALQR.G (2+) R.TNGAIGDGVTDDTAALQR.G (3+) R.GINNAASSGQVFFLDAGTYK.V (3+) R.GINNAASSGQVFFLDAGTYK.V (2+) K.VTSTITIPPGSR.I (2+) R.IVGESYSTIMSSGNFFNNINSPQPVVR.V (3+) R.IGGFTGSNLQVAQCEK.N (3+) R.IGGFTGSNLQVAQCEK.N (2+) R.QLDIYAGR.G (2+) R.NGQSQALYNANLNVYPTLITLYK.S (3+)
8	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) K.YDPSGPSDFQGAITPNNRPASLLSGNK.Y (3+) K.YYTQSKPQYNTLSVSSFTSAR.T (3+) R.TNGAIGDGVTDDTAALQR.G (2+) R.TNGAIGDGVTDDTAALQR.G (3+) R.GINNAASSGQVFFLDAGTYK.V (2+) R.IVGESYSTIMSSGNFFNNINSPQPVVR.V (3+)

Table S6-1. Continued.

8	XP_001588171	R.SVDSFIATESPIAMR.N (3+) K.VNPDYFYTWTR.D (2+) K.WLIANGYTSTVQTIVWPIIR.N (3+) K.QGSITVTSTSLAFFR.D (2+) R.AGGAPLSAYDLTWSYAAFLTAAAR.R (3+) K.IAGSISQLGSWAPASAVALSASK.Y (2+) K.IAGSISQLGSWAPASAVALSASK.Y (3+) K.YTSSNPLWSVTINLPAGTAIQYK.F (3+) K.FINVASDGTVTWEADPNR.S (2+) K.FINVASDGTVTWEADPNR.S (3+)
8	XP_001588171	K.VNPDYFYTWTR.D (2+) R.DFSSSVTAGTYSSSTSTYTTLYNAIK.T (3+) K.IAGSISQLGSWAPASAVALSASK.Y (2+) K.YTSSNPLWSVTINLPAGTAIQYK.F (3+) K.FINVASDGTVTWEADPNR.S (3+)
8	XP_001591947	K.DFGAVGDGVTDDTAAINAAISAGNR.C (3+) R.QQYFTQLVGDAITLPTIK.G (3+) R.AAGSATIMDSTITNTPIGISTVYDIK.E (3+) K.GDGVTDDTAAIQAIFDK.A (3+)
8	XP_001591947	K.DFGAVGDGVTDDTAAINAAISAGNR.C (3+) R.QQYFTQLVGDAITLPTIK.G (3+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) K.GDGVTDDTAAIQAIFDK.A (2+) K.ATTDQIVYFDHGGYVVTDTIK.V (3+)
8	XP_001590395	R.AAGIDNYLTSESPIALQGVLNNIGPNGSK.A (3+) K.LQTVENPSGDLDSGAGLAEPK.F (3+) R.SIYTVNSDAPAGQAVAIGR.Y (3+)
8	XP_001590395	K.APGANAGIVVASPSTVDPNYFYTWTR.D (3+) K.LQTVENPSGDLDSGAGLAEPK.F (3+) K.AMQDYADGFVAVAEK.Y (2+)
8	XP_001593684	R.YYDLTVSR.S (2+) K.NVILINDQFPGPLIEANWGDVINVR.V (3+) R.GTWDGSITNPSNPLR.R (2+)
8	XP_001593684	K.NVILINDQFPGPLIEANWGDVINVR.V (3+) R.ANYNSPLLLLASEGTPLAPLPK.D (3+)
8	XP_001594665	K.EFLQLGASSDGQFTTASTAGVINNIDGR.E (3+) R.ALLPTQIDDMFLESDIYSPTGTTYR.I (3+) R.IGTDDLAQHITFMR.T (3+) K.EITWNQAWLAATGIANAEK.F (3+)
8	XP_001594665	K.EFLQLGASSDGQFTTASTAGVINNIDGR.E (3+) R.IGTDDLAQHITFMR.T (3+)
9	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) R.NLVFDMTGIPAGTTATGLHWPTSQATSLQNVEFR.M (3+) R.KYDPSGPSDFQGAITPNNRPASLLSGNK.Y (3+) K.YDPSGPSDFQGAITPNNRPASLLSGNK.Y (3+) K.YYTQSKPQYNTLSVSSFTSAR.T (3+) K.YYTQSKPQYNTLSVSSFTSAR.T (2+) R.TNGAIGDGVTDDTAALQR.G (3+) R.TNGAIGDGVTDDTAALQR.G (2+) R.GINNAASSGQVFFLDAGTYK.V (2+) R.GINNAASSGQVFFLDAGTYK.V (3+) K.VTSTITIPPGSR.I (2+) R.IVGESYSTIMSSGNFFNNINSPQPVVR.V (3+) R.IGGFTGSNLQVAQCEK.N (2+) R.QLDIYAGR.G (2+) R.NGQSQALYNANLNVYPTLITLYK.S (2+) R.NGQSQALYNANLNVYPTLITLYK.S (3+)
9	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) R.NLVFDMTGIPAGTTATGLHWPTSQATSLQNVEFR.M (3+) R.KYDPSGPSDFQGAITPNNRPASLLSGNK.Y (3+) K.YDPSGPSDFQGAITPNNRPASLLSGNK.Y (3+) R.TNGAIGDGVTDDTAALQR.G (2+) R.GINNAASSGQVFFLDAGTYK.V (3+) R.GINNAASSGQVFFLDAGTYK.V (2+) R.IVGESYSTIMSSGNFFNNINSPQPVVR.V (3+) R.IGGFTGSNLQVAQCEK.N (3+) R.IGGFTGSNLQVAQCEK.N (2+) R.NGQSQALYNANLNVYPTLITLYK.S (2+) R.NGQSQALYNANLNVYPTLITLYK.S (3+)
9	XP_001591947	R.QGAPAYGDASYQIFR.N (2+) K.DFGAVGDGVTDDTAAINAAISAGNR.C (3+) R.QQYFTQLVGDAITLPTIK.G (3+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) R.TKPQYETLPASSFLSVK.S (3+) K.GDGVTDDTAAIQAIFDK.A (2+) K.GDGVTDDTAAIQAIFDK.A (3+)
9	XP_001591947	R.AAGSATIMDSTITNTPIGISTVYDIK.E (3+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) R.TKPQYETLPASSFLSVK.S (3+) K.GDGVTDDTAAIQAIFDK.A (2+) K.GDGVTDDTAAIQAIFDK.A (3+) K.ATTDQIVYFDHGGYVVTDTIK.V (3+)
9	XP_001590395	R.AAGIDNYLTSESPIALQGVLNNIGPNGSK.A (3+) K.LQTVENPSGDLDSGAGLAEPK.F (3+) R.ATALISFGNDR.L (2+) K.SDSTQYADIVK.A (2+) K.YTPEDGALAEQFSR.E (2+)
9	XP_001590395	R.AAGIDNYLTSESPIALQGVLNNIGPNGSK.A (3+) K.APGANAGIVVASPSTVDPNYFYTWTR.D (3+) K.LQTVENPSGDLDSGAGLAEPK.F (3+) K.AMQDYADGFVAVAEK.Y (2+)
9	XP_001556598	K.VTSTITIPPGSR.I (2+) R.IGGFTGSNLQVAQCEK.N (2+) R.NGQSQAVYSDNINVYPTLITLYK.S (2+) R.NGQSQAVYSDNINVYPTLITLYK.S (2+)
9	XP_001556598	R.IGGFTGSNLQVAQCEK.N (3+) R.IGGFTGSNLQVAQCEK.N (2+) R.NGQSQAVYSDNINVYPTLITLYK.S (2+)
9	XP_001588171	K.IAGSISQLGSWAPASAVALSASK.Y (3+) K.YTSSNPLWSVTINLPAGTAIQYK.F (3+) K.FINVASDGTVTWEADPNR.S (3+)
9	XP_001588171	R.NLLCNIGSSGSCAAGASSGIVIASPDK.V (3+) K.VNPDYFYTWTR.D (2+) K.YTSSNPLWSVTINLPAGTAIQYK.F (3+) K.FINVASDGTVTWEADPNR.S (3+)
9	XP_001554182	R.TKPQYETLPASSFLSVK.S (3+) K.GDGTTDDTAAIQAIFDK.A (2+) K.GDGTTDDTAAIQAIFDK.A (3+)
9	XP_001554182	R.TKPQYETLPASSFLSVK.S (3+) K.GDGTTDDTAAIQAIFDK.A (2+) K.GDGTTDDTAAIQAIFDK.A (3+)

Table S6-1. Continued.

10	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) K.YYTQSKPQYNTLSVSSFTSAR.T (3+) R.TNGAIGDGVTDDTAALQR.G (3+) R.TNGAIGDGVTDDTAALQR.G (2+) R.GINNAASSGQVFFLDAGTYK.V (2+) K.VTSTITIPPGSR.I (2+) R.IGGFTGSNLQVAQCEK.N (2+) R.QLDIYAGR.G (2+) R.NGQSQALYNANLNVYPTLITLYK.S(3+)
10	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) K.YDPSGPSDFQGAITPNNRPASLLSGNK.Y (3+) K.YYTQSKPQYNTLSVSSFTSAR.T (3+) R.TNGAIGDGVTDDTAALQR.G (2+) R.GINNAASSGQVFFLDAGTYK.V (2+) R.IGGFTGSNLQVAQCEK.N (2+) R.IGGFTGSNLQVAQCEK.N (3+) R.NGQSQALYNANLNVYPTLITLYK.S (3+)
10	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.NIDLEILVK.N (2+) K.SSQIFNVGTNSIVK.E (2+) K.APTLEALLYDPTK.A (2+) R.FSTLATSTIPR.L (2+) K.LKPDAADEFVTEFR.V (3+)
10	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.NIDLEILVK.N (2+) R.IQPLSANPTWELDSMPEGR.G (3+) K.APTLEALLYDPTK.A (2+) K.LKPDAADEFVTEFR.V (3+)
10	XP_001594660	K.EAFTFAWDGYQK.Y (2+) K.YAFPHDELHPIANSYSDSR.N (3+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+)
10	XP_001594660	K.EAFTFAWDGYQK.Y (2+) K.YAFPHDELHPIANSYSDSR.N (3+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) R.TGSGFAELENVNAPNGGGFNDFQDSFLFAEVMK.Y (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+)
10	XP_001589330	K.LAGVEDADVCAGAIGLEGPIIAHDLR.Q (3+) K.AFIDPTAASSADSFGAYSTLLPGSNLR.I (3+) R.IISINTNLYYR.S (2+) R.SNYWLYEATMEK.D (2+)
10	XP_001589330	K.GVAHLGNDAFVDTLTEICK.L (3+) K.LAGVEDADVCAGAIGLEGPIIAHDLR.Q (3+) K.AFIDPTAASSADSFGAYSTLLPGSNLR.I (3+) R.IISINTNLYYR.S (2+)
10	XP_001590395	R.AAGIDNYLTSESPIALQGVLNNIGPNGSK.A (3+) K.LQTVENPSGDLDSGAGLAEPK.F (3+) K.SDSTQYADIVK.A (2+) K.YTPEDGALAEQFSR.E (2+)
10	XP_001590395	R.AAGIDNYLTSESPIALQGVLNNIGPNGSK.A (3+) K.APGANAGIVVASPSTVDPNYFYTWTR.D (3+) K.LQTVENPSGDLDSGAGLAEPK.F (3+) K.AMQDYADGFVAVAEK.Y (2+)
10	XP_001586988	R.DIPILQELGTNVIR.V (2+) R.TIGVGYATNDDANIR.I (2+) R.TIGVGYATNDDANIR.I (3+)
10	XP_001586988	R.DIPILQELGTNVIR.V (2+) R.DIPILQELGTNVIR.V (3+) R.TIGVGYATNDDANIR.I (2+)
11	XP_001594660	K.EAFTFAWDGYQK.Y (3+) K.EAFTFAWDGYQK.Y (2+) K.YAFPHDELHPIANSYSDSR.N (3+) R.YVGGLLAGYDLLK.G (2+) R.YVGGLLAGYDLLK.G (3+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) K.MYVYDSTR.F (2+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+) K.WVFNTEAHPLK.V (3+) K.WVFNTEAHPLK.V (2+)
11	XP_001594660	K.EAFTFAWDGYQK.Y (2+) K.EAFTFAWDGYQK.Y (3+) K.YAFPHDELHPIANSYSDSR.N (3+) R.YVGGLLAGYDLLK.G (3+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) R.LFVSEHLACFDGGNFLLAGLVLK.E (3+) K.AGFYITNSDYILRPEVLESFYYAYR.V (3+) K.YAYLIHGDDEEYQVGGGGVNK. (3+) K.WVFNTEAHPLK.V (3+)
11	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) K.YYTQSKPQYNTLSVSSFTSAR.T (3+) R.TNGAIGDGVTDDTAALQR.G (3+) R.TNGAIGDGVTDDTAALQR.G (2+) R.IGGFTGSNLQVAQCEK.N (2+) R.QLDIYAGR.G (2+) R.NGQSQALYNANLNVYPTLITLYK.S (3+)
11	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) K.YYTQSKPQYNTLSVSSFTSAR.T (3+) R.GINNAASSGQVFFLDAGTYK.V (3+) R.IVGESYSTIMSSGNFFNNINSPQPVVR.V (3+)
11	XP_001589330	K.GVAHLGNDAFVDTLTEICK.L (3+) K.LAGVEDADVCAGAIGLEGPIIAHDLR.Q (3+) K.AFIDPTAASSADSFGAYSTLLPGSNLR.I (3+) R.IISINTNLYYR.S (2+)
11	XP_001589330	K.SIESATTCAGCNAVLLLLK.G (2+) K.SIESATTCAGCNAVLLLLK.G (3+) K.GVAHLGNDAFVDTLTEICK.L (3+) K.LAGVEDADVCAGAIGLEGPIIAHDLR.Q (3+) K.CDATLSLEESMYAAIK.E (3+) K.AFIDPTAASSADSFGAYSTLLPGSNLR.I (3+) R.IISINTNLYYR.S (2+) R.IISINTNLYYR.S (3+) R.SNYWLYEATMEK.D (2+) R.GYNVETCSGDCATQEICQIR.A (3+)
11	XP_001586988	R.DIPILQELGTNVIR.V (2+) R.DIPILQELGTNVIR.V (3+) R.TIGVGYATNDDANIR.I (2+) R.TIGVGYATNDDANIR.I (3+)
11	XP_001586988	R.DIPILQELGTNVIR.V (2+) R.DIPILQELGTNVIR.V (3+)R.TIGVGYATNDDANIR.I (2+) R.TIGVGYATNDDANIR.I (3+)
11	XP_001594345	R.GVINLQAR.Y (2+) R.ETVSATETLANTGAGNPVQEGTGLVR.S (3+) K.YGIVHDPIYGQVYAYEVDGFGSSNR.M (3+)
11	XP_001594345	R.DSANQMQSYLPLLTK.N (2+) R.ETVSATETLANTGAGNPVQEGTGLVR.S (3+) R.SAFRPSDDATIYQLFIPANMMFSR.Y (3+) K.YGIVHDPIYGQVYAYEVDGFGSSNR.M (3+) R.ILTTSDPDEIYSTLK.E (2+) R.ILTTSDPDEIYSTLK.E (3+)
12	XP_001594660	K.EAFTFAWDGYQK.Y (2+) K.EAFTFAWDGYQK.Y (3+) K.YAFPHDELHPIANSYSDSR.N (3+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) K.MYVYDSTR.F (2+) K.AGFYITNSDYILRPEVLESFYYAYR.V (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (2+) K.WVFNTEAHPLK.V (2+)

Table S6-1. Continued.

12	XP_001594660	K.EAFTFAWDGYQK.Y (2+) K.EAFTFAWDGYQK.Y (3+) K.YAFPHDELHPIANSYSDSR.N (3+) R.YVGGLLAGYDLLK.G (3+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) K.AGFYITNSDYILRPEVLESFYYAYR.V (3+) R.TGSGFAELENVNAPNGGGFNDFQDSFLFAEVMK.Y (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+)
12	XP_001594345	K.SVIADPDLYR.I (2+) R.ETVSATETLANTGAGNPVQEGTGLVR.S (3+) R.ILTTSDPDEIYSTLK.E (2+)
12	XP_001594345	R.ETVSATETLANTGAGNPVQEGTGLVR.S (3+) K.YGIVHDPIYGQVYAYEVDGFGSSNR.M (3+) R.ILTTSDPDEIYSTLK.E (2+)
12	XP_001586988	R.TIGVGYATNDDANIR.I (2+)
12	XP_001586988	R.DIPILQELGTNVIR.V (2+) R.TIGVGYATNDDANIR.I (2+) R.TIGVGYATNDDANIR.I (3+)
13	XP_001594660	K.EAFTFAWDGYQK.Y ((2+) K.YAFPHDELHPIANSYSDSR.N (3+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+) K.WVFNTEAHPLK.V (3+)
13	XP_001594660	K.EAFTFAWDGYQK.Y (2+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) K.TRLFVSEHLACFDGGNFLLAGLVLK.E (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+)
13	XP_001596243	K.AYGVFISPGTGYR.N (2+) R.FLTAIVK.G (2+) K.ANQWALR.G (2+) K.YATTSLTSGPGLTVGSSISLR.A (2+) K.YATTSLTSGPGLTVGSSISLR.A (3+) R.YIAHTGSTVNTQVVTTSSATALK.Q (3+) K.QQSSWYVR.T (2+) R.TGLGNSACFSFESNDTPGSYIR.H (3+)
13	XP_001596243	R.YIAHTGSTVNTQVVTTSSATALK.Q (3+) R.HYNFGLLLNANDGSK.Q (3+)
13	XP_001591275	K.IAEEGIHEYNHPTR.V (3+) R.LVLGDVVPETAIPMSLTPPTYK.K (3+) K.NHVATFGISDEEAAPHIK.K (3+) R.SDYDAFIK.N (2+) K.NGIPGGGIATGAEGVK.T (2+) R.LIAHSVATYAISFDGFPK.R (3+)
13	XP_001591275	R.LVLGDVVPETAIPMSLTPPTYK.K (3+) K.AGALAAVIYNNAHGALGGTLGAPDK.N (3+) K.HVDGSAYIDAVVQAIETTNIVAQTK.G (3+)
13	XP_001596362	K.SSQIFNVGTNSIVK.E (2+) K.ELPELAGDYR.T(2+) K.APTLEALLYDPTK.A (2+)
13	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) R.IQPLSANPTWELDSMPEGR.G (3+) K.APTLEALLYDPTK.A (2+) K.ADGSTLDVTFNCPAGAK.A (2+) R.MLHLDNTGFVAGATQQK.L (2+)
13	XP_001589422	R.QVEFYFDTAR.T (2+) R.YQVAHVGDSFPSVIDR.F (3+)
13	XP_001589422	R.VLSITTPGSTVYSGDR.E (2+) R.LLNEWQAALVR.T (2+) R.QVEFYFDTAR.T (2+) R.YQVAHVGDSFPSVIDR.F (3+) R.FWWDTAGPTFPHQVLGLVAYDVPK.T (3+) K.TQLTFGTDWPFR.R (2+)
14	XP_001594757	K.NVVAMTDFLENQLK.A (2+) R.DFQDTIGSR.A (2+) R.FPSLTIHGITGADSSPNQTTSIAPSVTAK.F (3+) R.VELFGESAPYWLGTSDDANFR.A (3+) R.EGGSIGVTLDLQNVLGEK.S (2+)
14	XP_001589422	R.VLSITTPGSTVYSGDR.E (2+) R.VLSITTPGSTVYSGDR.E (3+) R.LLNEWQAALVR.T (2+) R.QVEFYFDTAR.T (2+)
14	XP_001589422	R.QIVPTIAGQPTPFWSIEAEFEFMK.S (3+) R.VLSITTPGSTVYSGDR.E (2+) R.LLNEWQAALVR.T (2+) K.QFSFVATIPLPYTEESIVEATYSISK.L (3+) R.QVEFYFDTAR.T (2+) R.YQVAHVGDSFPSVIDR.F (3+) R.FWWDTAGPTFPHQVLGLVAYDVPK.T (3+) K.TQLTFGTDWPFR.R (2+) R.NHEANVEAMLNAPFLNEDDLDWILAK.S (3+)
14	XP_001590904	K.VASDEDVSTVLK.I (2+) R.FGIVTSVVLK.T (2+) R.GLLQQTISHLTDVAK.S (3+)
14	XP_001590904	R.FGIVTSVVLK.T (2+) K.SPIPLNVYFAWTSPSDDAYWR.G (3+) R.EKYDPDGVMLLAGGFDI.- (2+)
14	XP_001591015	R.VFSILLGDAIPK.D (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (3+)
14	XP_001591015	R.VFSILLGDAIPK.D (2+) R.FDVGANVFSYVAQLTGDK.V (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (3+) K.DTIYQGQLEIPDGLNFEPNA.- (3+)
15	XP_001591015	K.GITWGLYQEDMPYTGYQGMDWR.N (3+) R.NEVTGANAYVR.K (2+) R.VFSILLGDAIPK.D (2+) R.FDVGANVFSYVAQLTGDK.V (2+) R.FDVGANVFSYVAQLTGDK.V (3+) R.SWTDGALESR.F (2+)
15	XP_001591015	R.VFSILLGDAIPK.D (3+) R.VFSILLGDAIPK.D (2+) R.FDVGANVFSYVAQLTGDK.V (2+) R.FDVGANVFSYVAQLTGDK.V (3+) R.FDVGANVFSYVAQLTGDKVR.S (3+) K.DKDTIYQGQLEIPDGLNFEPNA.- (3+) K.DTIYQGQLEIPDGLNFEPNA.- (3+)
15	XP_001589422	R.VLSITTPGSTVYSGDR.E (2+) R.LLNEWQAALVR.T (2+) R.QVEFYFDTAR.T (2+) R.YQVAHVGDSFPSVIDR.F (3+) R.FWWDTAGPTFPHQVLGLVAYDVPK.T (3+) K.TQLTFGTDWPFR.R (2+) R.RLYDIGPL.- (2+)
15	XP_001589422	R.QIVPTIAGQPTPFWSIEAEFEFMK.S (3+) R.VLSITTPGSTVYSGDR.E (2+) R.LLNEWQAALVR.T (2+) K.QFSFVATIPLPYTEESIVEATYSISK.L (3+) R.QVEFYFDTAR.T (2+) R.YQVAHVGDSFPSVIDR.F (3+) R.FWWDTAGPTFPHQVLGLVAYDVPK.T (3+) K.TQLTFGTDWPFR.R (2+)
15	XP_001597582	R.SQSIYQVITDR.F (3+) R.TDGSTTASCNVNEYCGGSWQGIIK.H (3+) R.FIASVNQIR.N (2+) R.NQAIYVDPTYLTYK.A (2+) K.QIIAVFSNK.G (2+)

Table S6-1. Continued.

15	XP_001597582	R.TDGSTTASCNVNEYCGGSWQGIIK.H (3+) K.SSCSDTTLLGSFLENHDVAR.F (3+) K.NAIAFTILSDGIPIVYQGQEQHLTGSSVPNNR.E (3+) R.EALWSNSNSYSQSATYYR.F (3+) R.NQAIYVDPTYLTYK.A (2+) K.AYPVYSDGTTIVMR.K (2+) K.SYLVGSGVCSL.- (2+)
15	XP_001596398	K.FNSYLLTLQSSGDLGPNR.V (3+) K.DTDGTGYLLTEDR.A (2+) K.SGAGNVIEFESYNSGWAPDIDR.I (3+)
15	XP_001596398	K.YVMWMHIDSSNYGEAR.A (3+) R.AGVATSSSVCGSYTYLGASQPLGYQSR.D (3+) R.YANVVVNGVSNIIAFLPTADGNTPGTSVLTTALK.S (3+)
16	XP_001589422	K.EILEAAQR.D (2+) R.VLSITTPGSTVYSGDR.E (2+) R.VLSITTPGSTVYSGDR.E (3+) R.LLNEWQAALVR.T (2+) R.HTIFVHPHTPYLR.Y (3+) R.YNGVLIEANPTIYPPR.Q (3+) R.QVEFYFDTAR.T (2+) R.YQVAHVGDSFPSVIDR.F (2+) R.YQVAHVGDSFPSVIDR.F (3+) R.FLSSSAENASDIYHTLQTR.F (3+) R.FWWDTAGPTFPHQVLGLVAYDVPK.T (3+) K.TQLTFGTDWPFR.R (2+) R.RLYDIGPL.- (2+)
16	XP_001589422	R.VLSITTPGSTVYSGDR.E (2+) R.LLNEWQAALVR.T (2+) R.QVEFYFDTAR.T (2+) R.FWWDTAGPTFPHQVLGLVAYDVPK.T (3+) K.TQLTFGTDWPFR.R (2+)
16	XP_001597382	R.ADTFAITGVQDGGIQPR.L (2+) R.NGYPYSVYDTTLR.W (2+) R.WPTSDTAAATSQNDQVESQLEANLR.N (3+) R.LLAIWQAVYPDSYVETAR.Q (3+) R.LLAIWQAVYPDSYVETAR.Q (2+) R.QGAATWTISR.G (2+) R.GSYQDQNSPLTPFHQDTNGK.F (3+) K.FFSPADVR.N (2+)
16	XP_001597382	R.ADTFAITGVQDGGIQPR.L (2+) R.ADTFAITGVQDGGIQPR.L (3+) R.NGYPYSVYDTTLR.W (2+) R.NGYPYSVYDTTLR.W (3+) R.WPTSDTAAATSQNDQVESQLEANLR.N (3+) R.LLAIWQAVYPDSYVETAR.Q (2+) R.LLAIWQAVYPDSYVETAR.Q (3+)
16	XP_001591015	K.GITWGLYQEDMPYTGYQGMDWR.N (3+) R.NEVTGANAYVR.K (2+) R.VFSILLGDAIPK.D (2+) R.FDVGANVFSYVAQLTGDK.V (2+) R.FDVGANVFSYVAQLTGDK.V (3+) R.SWTDGALESR.F (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (3+)
16	XP_001591015	K.GITWGLYQEDMPYTGYQGMDWR.N (3+) R.VFSILLGDAIPK.D (2+) R.FDVGANVFSYVAQLTGDK.V (2+)
17	XP_001596115	R.FAADPEDDDR.A (2+) R.SLGILTTDPLSTDLGK.E (2+) K.TDCVYFR.D (2+) R.NIVDMAAQIR.I (2+) K.WIGDFHDENNKPNPDQAK.L (3+) K.EGIQFVVDEELR.I (2+) K.EGIQFVVDEELR.I (3+) K.VNATSLEAQLIR.E (2+) K.VNATSLEAQLIR.E (3+) R.AELDEYQAR.I (2+) K.IQDVMDEYEAK.Q (2+) R.TLELLQGAWK.S (2+) K.SMVTDLETIK.Q (2+) K.IVNEWNELR.E (2+)
17	XP_001596115	R.SLGILTTDPLSTDLGK.E (2+) K.EGIQFVVDEELR.I (2+)
17	XP_001597382	R.ADTFAITGVQDGGIQPR.L (2+) R.ADTFAITGVQDGGIQPR.L (3+) R.NGYPYSVYDTTLR.W (2+) R.WPTSDTAAATSQNDQVESQLEANLR.N (3+) R.LLAIWQAVYPDSYVETAR.Q (3+) R.QGAATWTISR.G (2+) K.FFSPADVR.N (2+)
17	XP_001597382	R.ADTFAITGVQDGGIQPR.L (2+) R.ADTFAITGVQDGGIQPR.L (3+) R.NGYPYSVYDTTLR.W (2+) R.WPTSDTAAATSQNDQVESQLEANLR.N (3+) R.TQIMTLFANWQPYNHFSNK.G (3+) R.LLAIWQAVYPDSYVETAR.Q (3+) R.LLAIWQAVYPDSYVETAR.Q (2+) R.GSYQDQNSPLTPFHQDTNGK.F (3+)
17	XP_001593115	R.KGPITWTQGDTTK.Q (3+) K.GPITWTQGDTTK.Q (2+) K.QTLAAIQTLAYR.Y (2+) K.QFYYDGWGNVR.N (2+) K.DVGTVDGLLAIDK.V (2+)
17	XP_001593115	K.IILDVHGAPGSQNGFDNSGR.K (3+) K.QTLAAIQTLAYR.Y (2+) R.YAPATDVVTGIELLNEPANWALDMGAVK.Q (3+) K.DVGTVDGLLAIDK.V (2+)
17	XP_001588805	K.GNAFFIGDNR.F (2+) K.IVDPIADKDTCR.R (3+) R.SIPVGYSAADVDSNR.L (2+) R.SIPVGYSAADVDSNR.L (3+) K.SLYSTDMTAVYSGGLVYEYSEEGSK.Y (3+) K.YGLVTINGGSVTEGPDFTALK.A (3+) K.YGLVTINGGSVTEGPDFTALK.A (2+) K.AAFEGTTNPSGDGGYSSTNK.A (3+) K.AAFEGTTNPSGDGGYSSTNK.A (2+)
17	XP_001588805	R.SIPVGYSAADVDSNRLEMAQYMNCGTDDER.S (3+) R.LEMAQYMNCGTDDER.S (2+) R.SDFFAFNDYSWCDPSSFTISGWDQK.V (3+) K.SLYSTDMTAVYSGGLVYEYSEEGSK.Y (3+) K.YGLVTINGGSVTEGPDFTALK.A (2+)
17	XP_001595540	K.TFFDNAMSQGLAMPVFTADLR.K (3+) K.ASAGSYEFGNIDSSK.F (2+) K.VQGAVNDQSVGGVTFPCSAK.L (3+) R.GDDINYAPVDNTGK.T (2+)
17	XP_001595540	K.TFFDNAMSQGLAMPVFTADLR.K (3+) K.LPDLAVDVGGNYMAVVR.G (2+) K.TCFGGLQATTSNLQIYGDIMFK.S (3+)
17	XP_001588269	K.YVLDHAGISALLR.F (3+) K.ELVDAQVAAIEALNQSAQK.A (3+) K.ILVADLTLIK.A (2+)
17	XP_001588269	K.ELVDAQVAAIEALNQSAQK.A (3+) K.IITDALSGDSGDIVK.L (2+)
18	XP_001588805	K.GNAFFIGDNR.F (2+) K.IVDPIADK.D (2+) R.SIPVGYSAADVDSNR.L (2+) R.SIPVGYSAADVDSNR.L (3+) K.SLYSTDMTAVYSGGLVYEYSEEGSK.Y (3+) K.YGLVTINGGSVTEGPDFTALK.A (2+) K.AAFEGTTNPSGDGGYSSTNK.A (2+) K.AAFEGTTNPSGDGGYSSTNK.A (3+)

Table S6-1. Continued.

18	XP_001588805	R.SIPVGYSAADVDSNR.L (2+) R.SIPVGYSAADVDSNR.L (3+) R.LEMAQYMNCGTDDER.S (2+) R.SDFFAFNDYSWCDPSSFTISGWDQK.V (3+) K.SLYSTDMTAVYSGGLVYEYSEEGSK.Y (3+) K.YGLVTINGGSVTEGPDFTALK.A (3+) K.YGLVTINGGSVTEGPDFTALK.A (2+)
18	XP_001597382	R.ADTFAITGVQDGGIQPR.L (2+) R.ADTFAITGVQDGGIQPR.L (3+) R.WPTSDTAAATSQNDQVESQLEANLR.N (3+) R.LLAIWQAVYPDSYVETAR.Q (2+) R.QGAATWTISR.G (2+) K.FFSPADVR.N (2+)
18	XP_001597382	R.WPTSDTAAATSQNDQVESQLEANLR.N (3+) R.LLAIWQAVYPDSYVETAR.Q (3+)
18	XP_001593115	K.GLSFDYNGDK.V (2+) K.QTLAAIQTLAYR.Y (2+) R.YAPATDVVTGIELLNEPANWALDMGAVK.Q (3+) K.QFYYDGWGNVR.N (2+) R.YDGSYPGSPAVYGSCQTK.D (3+) K.DVGTVDGLLAIDK.V (2+)
18	XP_001593115	K.QTLAAIQTLAYR.Y (2+) K.QTLAAIQTLAYR.Y (3+) K.WTIVGEFSGAQTDCAK.W (2+) K.WTIVGEFSGAQTDCAK.W (3+) R.YDGSYPGSPAVYGSCQTK.D (2+) R.YDGSYPGSPAVYGSCQTK.D (3+) K.DVGTVDGLLAIDK.V (2+) K.DVGTVDGLLAIDK.V (3+)
18	XP_001593425	R.VQSDFQAAFEAANSLVGTSGFTSAR.L (3+) R.VVGISVGSEDLYR.N (2+) K.AGYGANPADLVDYIK.Q (2+) K.SLFNAALAATQSATGK.T (2+) K.TVWVTETGWPVTGATAGDGIPSAANAK.T (3+)
18	XP_001593425	R.VQSDFQAAFEAANSLVGTSGFTSAR.L (3+) R.VVGISVGSEDLYR.N (2+) K.SLFNAALAATQSATGK.T (2+)
18	XP_001588269	K.IITDALSGDSGDIVK.L (2+) K.IITDALSGDSGDIVK.L (3+) K.ILVADLTLIK.A (2+) K.INQNSIITK.W (2+)
18	XP_001588269	K.ELVDAQVAAIEALNQSAQK.A (3+) K.IITDALSGDSGDIVK.L (2+) K.ILVADLTLIK.A (2+)
18	XP_001593876	K.GIGGYQVPAPGDTDHQFIAPK.S (3+) K.LSIGCDATSR.T (2+) K.NYVPDYETISSFFGAVK.N (2+) K.NYVPDYETISSFFGAVK.N (3+)
18	XP_001593876	R.GPCPGLNAAANHNFLAR.D (3+) K.LNPLFGNLGCPIALT.- (2+)
19	XP_001594755	M.PTYAPIVNELR.D (3+) M.PTYAPIVNELR.D (2+) M.PTYAPIVNELRDELLR.E (3+) K.YDIPDMVTYGIISGK.G (2+) K.GFLDFADWLVR.G (2+) R.GWIPTETTHGR.D (2+) K.NVGKPLTPLSDWLVR.Y (2+) K.NVGKPLTPLSDWLVR.Y (3+) R.FMDQPESWNETSLTSYR.N (3+) R.FMDQPESWNETSLTSYR.N (2+) R.VFEAEDPSNWK.T (2+) K.TFNQFFHR.K (2+) K.TFNQFFHR.K (3+) K.YGDLFK.E (2+) K.KQDPASVVTLDAPDTPGYQFLQTR.G (3+) K.QDPASVVTLDAPDTPGYQFLQTR.G (2+) K.QDPASVVTLDAPDTPGYQFLQTR.G (3+) R.GIIIINNDK.L (2+) R.VAILPIGMAVVSSVVMNTELLGK.T (3+)
19	XP_001594755	K.GFLDFADWLVR.G (2+) K.NVGKPLTPLSDWLVR.Y (3+) R.FMDQPESWNETSLTSYR.N (3+) K.KQDPASVVTLDAPDTPGYQFLQTR.G (3+) K.QDPASVVTLDAPDTPGYQFLQTR.G (3+)
19	XP_001588805	K.GNAFFIGDNR.F (2+) R.SIPVGYSAADVDSNR.L (2+) K.SLYSTDMTAVYSGGLVYEYSEEGSK.Y (3+) K.AAFEGTTNPSGDGGYSSTNK.A (2+) K.AAFEGTTNPSGDGGYSSTNK.A (3+)
19	XP_001588805	R.SIPVGYSAADVDSNR.L (2+) R.SIPVGYSAADVDSNRLEMAQYMNCGTDDER.S (3+) R.LEMAQYMNCGTDDER.S (2+) R.LEMAQYMNCGTDDER.S (3+) R.SDFFAFNDYSWCDPSSFTISGWDQK.V (3+) K.SLYSTDMTAVYSGGLVYEYSEEGSK.Y (3+) K.YGLVTINGGSVTEGPDFTALK.A (2+) K.YGLVTINGGSVTEGPDFTALK.A (3+)
19	XP_001593425	K.TAITQYGSAFTSR.V (2+) R.VVGISVGSEDLYR.N (2+) K.AGYGANPADLVDYIK.Q (2+) K.TVWVTETGWPVTGATAGDGIPSAANAK.T (3+)
19	XP_001593425	R.VQSDFQAAFEAANSLVGTSGFTSAR.L (3+) R.VVGISVGSEDLYR.N (2+) K.AGYGANPADLVDYIK.Q (2+) K.SLFNAALAATQSATGK.T (2+) K.TYWDDVGCPNFGK.I (2+)
19	XP_001591030	K.SDLTTFMNNYAYFANK.A (3+) K.AGLGFLNPWLYSTAK.S (2+) K.AGLGFLNPWLYSTAK.S (3+) K.SALTDITTGK.I (2+)
19	XP_001591030	K.SDLTTFMNNYAYFANK.A (2+) K.SDLTTFMNNYAYFANK.A (3+) R.GVSILVASGDSGVGTTCTTTGSTK.G (2+) K.AGLGFLNPWLYSTAK.S (2+) K.AGLGFLNPWLYSTAK.S (3+)
19	XP_001586339	R.LTAFQTGGGGSSTPPSNPAPSK.S (3+) K.TVDQNWVTAAQSAK.S (2+) R.IGGPGVLWNNWNSWSSGGGYSSR.V (3+) R.TVLPWLDSQGYVER.Y (2+) R.TVLPWLDSQGYVER.Y (3+)
19	XP_001586339	R.EGWTSAGCYTDNVSGR.A (3+) R.EGWTSAGCYTDNVSGR.A (2+) K.SVLGFNEPDLTYEQSSNMLPEVAAQGYK.S (3+) K.SWIQPFAGQVR.I (2+) R.IGGPGVLWNNWNSWSSGGGYSSR.V (3+) R.TVLPWLDSQGYVER.Y (2+)
19	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) R.GINNAASSGQVFFLDAGTYK.V (3+)
19	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) R.GINNAASSGQVFFLDAGTYK.V (3+) R.IVGESYSTIMSSGNFFNNINSPQPVVR.V (3+)
20	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) K.GDNPIWEDAK.V (2+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.DPWHTETILEEK.W (2+) K.DPWHTETILEEK.W (3+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (2+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (2+) R.NLNPVNEVGTLENENGSWK.H (3+)

Table S6-1. Continued.

20	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) R.SPDGGNVWTTQDTIIAK.D (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.WGDELPGTALACVIGNGK.I (2+) K.DGSVHLFYVSK.S (3+) K.EEELINFFPGSK.L (2+) R.NLNPVNEVGTLENENGSWK.H (2+) R.NLNPVNEVGTLENENGSWK.H (3+)
20	XP_001593425	R.VQSDFQAAFEAANSLVGTSGFTSAR.L (3+) R.VVGISVGSEDLYR.N (2+) K.AGYGANPADLVDYIK.Q (2+) K.SLFNAALAATQSATGK.T (2+) K.SLFNAALAATQSATGK.T (3+)
20	XP_001593425	R.VQSDFQAAFEAANSLVGTSGFTSAR.L (3+) R.VVGISVGSEDLYR.N (2+) K.AGYGANPADLVDYIK.Q (2+) K.SLFNAALAATQSATGK.T (2+) K.TVWVTETGWPVTGATAGDGIPSAANAK.T (3+)
20	XP_001584844	K.SGLTIALTSTINTVFGSR.V (2+) K.SGLTIALTSTINTVFGSR.V (3+) R.IITEVIQHLWNVLDK.N (2+) R.IITEVIQHLWNVLDK.N (3+) R.QLNSAGYAI.- (2+)
20	XP_001584844	K.SGLTIALTSTINTVFGSR.V (2+) R.IITEVIQHLWNVLDK.N (3+)
20	XP_001591030	R.GVSILVASGDSGVGTTCTTTGSTK.G (3+) K.AGLGFLNPWLYSTAK.S (2+) K.AGLGFLNPWLYSTAK.S (3+) K.SALTDITTGK.I (2+)
20	XP_001591030	K.AGLGFLNPWLYSTAK.S (2+) K.AGLGFLNPWLYSTAK.S (3+) K.ITGCSGVISGAGFSAVSGWDPATGLGTPNYTK.L (3+)
21	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) R.SPDGGNVWTTQDTIIAK.D (3+) K.DGDYNGSSMTCYYVDK.D (3+) K.DANFDNQPSIHLLYMNK.D (3+) K.DANFDNQPSIHLLYMNK.D (2+) R.MKGDNPIWEDAK.V (2+) K.GDNPIWEDAK.V (2+) K.GDNPIWEDAKVPDEVR.K (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (2+) K.DPWHTETILEEK.W (2+) K.DPWHTETILEEK.W (3+) K.WGDELPGTALACVIGNGK.I (2+) K.DKVQATTPLACTMTK.D (2+) K.DKVQATTPLACTMTK.D (3+) K.VQATTPLACTMTK.D (3+) K.DGSVHLFYVSK.S (2+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (2+) K.KEEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (2+) K.EEELINFFPGSK.L (3+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (2+)
21	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) R.SPDGGNVWTTQDTIIAK.D (3+) K.DGDYNGSSMTCYYVDK.D (3+) K.DGDYNGSSMTCYYVDK.D (2+) K.GDNPIWEDAKVPDEVR.K (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.DPWHTETILEEK.W (2+) K.WGDELPGTALACVIGNGK.I (2+) K.WGDELPGTALACVIGNGK.I (3+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (3+) K.KEEELINFFPGSK.L (2+) K.EEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (2+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (2+) R.NLNPVNEVGTLENENGSWK.H (3+)
21	XP_001598370	R.LPFLFER.M (2+) K.EVVDYITAAGASAVLDAHNFGR.Y (3+) K.LAAVFATNDK.V (2+) R.IADATAWLK.A (2+)
21	XP_001598370	R.LPFLFER.M (2+) R.MAQGSLTATLDATYLASYK.E (2+) R.MAQGSLTATLDATYLASYK.E (3+) K.EVVDYITAAGASAVLDAHNFGR.Y (3+) R.YNGNIITSTSDFGSFWSK.L (2+) K.VIFDCNNEFHDEPTATIYAELNQACVTAVR.G (3+)
22	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) R.SPDGGNVWTTQDTIIAK.D (3+) K.DANFDNQPSIHLLYMNK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) R.MKGDNPIWEDAK.V (2+) K.GDNPIWEDAK.V (2+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.DPWHTETILEEK.W (2+) K.DPWHTETILEEK.W (3+) K.WGDELPGTALACVIGNGK.I (2+) K.WGDELPGTALACVIGNGK.I (3+) R.VFLQHHDYSIILYENQNNTWHDR.G (3+) K.DKVQATTPLACTMTK.D (3+) K.VQATTPLACTMTK.D (2+) K.DGSVHLFYVSK.S (2+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (2+) K.KEEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (2+) K.ITLFFR.N (2+)
22	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) R.SPDGGNVWTTQDTIIAK.D (3+) K.DGDYNGSSMTCYYVDK.D (2+) K.DGDYNGSSMTCYYVDK.D (3+) K.DANFDNQPSIHLLYMNK.D (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.DPWHTETILEEK.W (2+) K.DPWHTETILEEK.W (3+) K.WGDELPGTALACVIGNGK.I (3+) R.VFLQHHDYSIILYENQNNTWHDR.G (3+) K.KEEELINFFPGSK.L (2+) K.KEEELINFFPGSK.L (3+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (2+) R.NLNPVNEVGTLENENGSWK.H (3+)
22	XP_001595093	R.SLSGYSWDISYADGSGASGVVGTDTVTIGK.T (3+) K.YTGSLTYTSVSSGNGFWEFPSTSYK.V (3+) K.YTGSLTYTSVSSGNGFWEFPSTSYK.V (3+) K.SGPSLGFAPSVSA.- (2+)
22	XP_001595093	R.SLSGYSWDISYADGSGASGVVGTDTVTIGK.T (3+) K.YTGSLTYTSVSSGNGFWEFPSTSYK.V (3+) K.SGPSLGFAPSVSA.- (2+)
22	XP_001598370	K.EVVDYITAAGASAVLDAHNFGR.Y (3+) R.IADATAWLK.A (2+)
22	XP_001598370	R.LPFLFER.M (2+) R.MAQGSLTATLDATYLASYK.E (2+) R.MAQGSLTATLDATYLASYK.E (3+) K.EVVDYITAAGASAVLDAHNFGR.Y (3+) R.YNGNIITSTSDFGSFWSK.L (2+) R.YNGNIITSTSDFGSFWSK.L (3+)

Table S6-1. Continued.

23	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) R.SPDGGNVWTTQDTIIAK.D (3+) K.DANFDNQPSIHLLYMNK.D (3+) K.GDNPIWEDAK.V (2+) K.DPWHTETILEEK.W (2+) K.DPWHTETILEEK.W (3+) K.WGDELPGTALACVIGNGK.I (2+) K.DKVQATTPLACTMTK.D (3+) K.VQATTPLACTMTK.D (2+) K.DGSVHLFYVSK.S (2+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (2+) K.KEEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (2+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (2+)
23	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) R.SPDGGNVWTTQDTIIAK.D (3+) K.DGDYNGSSMTCYYVDK.D (3+) K.DANFDNQPSIHLLYMNK.D (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.DPWHTETILEEK.W (3+) K.WGDELPGTALACVIGNGK.I (2+) K.WGDELPGTALACVIGNGK.I (3+) K.DGSVHLFYVSK.S (3+) K.EEELINFFPGSK.L (2+) R.NLNPVNEVGTLENENGSWK.H (2+)
23	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) K.NTGTGYVEVHIASSSSNFQTR.I (2+) R.ILEVGTTFAQEDNGTWR.L (2+) K.SSNSVLPDLVYIK.T (3+) K.SSNSVLPDLVYIK.T (2+) K.VEVHIASGASTYK.T (2+) K.VEVHIASGASTYK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (2+) K.DANTGTGTTEVHIASR.A (3+) R.LLDVGSTFTQEQNGVWQLIDFNANGK.L (3+) K.LDLTYIK.Y (2+) K.YQNTGTGTVEVHVASG.- (2+) K.YQNTGTGTVEVHVASG.- (3+)
23	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) R.ILEVGTTFAQEDNGTWR.L (2+) K.SSNSVLPDLVYIK.T (3+) K.SSNSVLPDLVYIK.T (2+) K.VEVHIASGASTYK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.YQNTGTGTVEVHVASG.- (2+)
23	XP_001597228	K.TATCEAYVTANPSAFK.N (2+) K.TATCEAYVTANPSAFK.N (3+) K.NAYWIINSIK.V (2+)
23	XP_001597228	R.GEMDIIEGVNAGTTNQMTLHTSANCVMK.N (3+) K.QHNIVLDTTFCGDWAGSVWSSGGCAASTK.T (3+) K.TATCEAYVTANPSAFK.N (2+) K.TATCEAYVTANPSAFK.N (3+) K.NAYWIINSIK.V (2+) K.NAYWIINSIK.V (3+)
23	XP_001598370	K.EVVDYITAAGASAVLDAHNFGR.Y (3+)
23	XP_001598370	R.MAQGSLTATLDATYLASYK.E (2+) R.MAQGSLTATLDATYLASYK.E (3+) K.EVVDYITAAGASAVLDAHNFGR.Y (3+)
24	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) R.SPDGGNVWTTQDTIIAK.D (3+) K.DANFDNQPSIHLLYMNK.D (3+) K.DPWHTETILEEK.W (3+) K.DPWHTETILEEK.W (2+) K.WGDELPGTALACVIGNGK.I (2+) K.DKVQATTPLACTMTK.D (3+) K.DGSVHLFYVSK.S (2+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (3+) K.KEEELINFFPGSK.L (2+) K.EEELINFFPGSK.L (2+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (2+)
24	XP_001584968	K.DANFDNQPSIHLLYMNK.D (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.WGDELPGTALACVIGNGK.I (3+) K.KEEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (2+)
24	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) R.ILEVGTTFAQEDNGTWR.L (3+) R.ILEVGTTFAQEDNGTWR.L (2+) K.SSNSVLPDLVYIK.T (2+) K.SSNSVLPDLVYIK.T (3+) K.VEVHIASGASTYK.T (2+) K.VEVHIASGASTYK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (2+) K.DANTGTGTTEVHIASR.A (3+) R.LLDVGSTFTQEQNGVWQLIDFNANGK.L (3+) K.LDLTYIK.Y (2+) K.YQNTGTGTVEVHVASG.- (3+) K.YQNTGTGTVEVHVASG.- (2+)
24	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (2+) K.NTGTGYVEVHIASSSSNFQTR.I (3+) R.ILEVGTTFAQEDNGTWR.L (3+) R.ILEVGTTFAQEDNGTWR.L (2+) K.SSNSVLPDLVYIK.T (3+) K.SSNSVLPDLVYIK.T (2+) K.VEVHIASGASTYK.T (3+) R.TVEVVTSFGNEQDGQWNVYDYDGDGKPDLVFIK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.TSNTGTGTTELFVASASSNYQTR.L (2+) K.DANTGTGTTEVHIASR.A (3+) K.YQNTGTGTVEVHVASG.- (2+) K.YQNTGTGTVEVHVASG.- (3+)
25	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) K.DPWHTETILEEK.W (2+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (3+) K.ITLFFR.N (2+)
25	XP_001584968	K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.WGDELPGTALACVIGNGK.I (3+) R.NLNPVNEVGTLENENGSWK.H (3+)
25	XP_001586883	R.DQYTLSGTFPLIK.Y (2+) R.QPMVAWESLTPAAQSALENTDFGK.A (3+) K.NANFENNFVK.A (2+)
25	XP_001586883	R.DQYTLSGTFPLIK.Y (3+) R.DQYTLSGTFPLIK.Y (2+) K.YEGIWPLDHSCGLTSTQGGR.Q (3+) R.QPMVAWESLTPAAQSALENTDFGK.A (3+)
26	XP_001586352	R.MIQSFPGGFNSLR.V (2+) R.VYASSDCNTIANVVPAAIATGGK.V (3+) K.VLVGVWTEDAGHYDAEK.Q (3+) R.GDTDAFTLSQQIYDVR.G (2+)
26	XP_001586352	R.VYASSDCNTIANVVPAAIATGGK.V (3+) K.VLVGVWTEDAGHYDAEK.Q (3+) R.GDTDAFTLSQQIYDVR.G (3+)
27	XP_001594278	K.EGVSAYWFSMQVVNAAEGVSK.L (3+) R.TTYNFFENSSGFGTTSVDVR.V (3+) R.VTGLSGSTVIIK.N (2+) K.NVAVTPGLVVTASGNLK.S (2+) K.NVAVTPGLVVTASGNLK.S (3+)
27	XP_001594278	K.LAALSVGIIDITWDYVPCPITTPLQVHLK.E (3+) K.NVAVTPGLVVTASGNLK.S (3+)

Table S6-1. Continued.

27	XP_001593909	K.HFFDASGTPTFDLYFVNK.I (2+) K.HFFDASGTPTFDLYFVNK.I (3+) K.IGDVPAPEDADVGPANTGAVDWLALSAK.A (3+) K.SVGLSEVFR.V (2+) K.VGVESVPYAAQYWFYS.- (2+)
27	XP_001593909	K.CGTTPTLPVNGGASELPSPSGTLVYAALGR.G (3+) K.HFFDASGTPTFDLYFVNK.I (3+) K.IGDVPAPEDADVGPANTGAVDWLALSAK.A (3+) K.SVGLSEVFR.V (2+) K.VGVESVPYAAQYWFYS.- (2+) K.VGVESVPYAAQYWFYS.- (3+)
27	XP_001598009	K.QQDAFFGWNSVISTTNFEDK.G (3+) K.QQDAFFGWNSVISTTNFEDK.G (2+) K.QQDAFFGWNSVISTTNFEDKGPETVYAVGTFN.- (3+) K.GPETVYAVGTFN.- (2+)
27	XP_001598009	K.TSNDWQVNWGYNTDGDFAVMTIVDTVK.Q (3+) K.QQDAFFGWNSVISTTNFEDK.G (3+) K.GPETVYAVGTFN.- (2+)
28	XP_001589205	K.GVGTYEIVPYQAPSLNLNAWEGK.L (3+) K.GVGTYEIVPYQAPSLNLNAWEGK.L (2+) K.LEPGAVVR.T (2+) K.ENHIVSTPQISPTDPSAR.W (2+) K.ENHIVSTPQISPTDPSAR.W (3+) R.WTIKPATTHQYEVFTINNK.V (2+) R.WTIKPATTHQYEVFTINNK.V (3+) K.VSELGQLTVK.D (2+) K.DYSTHSGADVLSASAK.T (3+) K.DYSTHSGADVLSASAK.T (2+) K.WYFDAK.- (2+)
28	XP_001589205	K.WQVALVAGSGDSAEYLIINVHSGYFLTATK.E (3+) K.ENHIVSTPQISPTDPSAR.W (3+) K.VSELGQLTVK.D (2+) K.DYSTHSGADVLSASAK.T (3+)
28	XP_001589549	K.AEGLDGAIVTSLSLLLK.G (2+) K.AEGLDGAIVTSLSLLLK.G (3+) K.GHETFAAYVLQR.L (2+) K.GHETFAAYVLQR.L (3+) R.LPADLVADGQK.V (2+)
28	XP_001589549	K.AEGLDGAIVTSLSLLLK.G (2+) K.AEGLDGAIVTSLSLLLK.G (3+) K.GHETFAAYVLQR.L (3+)
29	XP_001595210	K.GGSELWTLLK.A (2+) R.SQNAGAPVESYLAVQDK.T (3+) R.SQNAGAPVESYLAVQDK.T (2+)
29	XP_001595210	K.GGSELWTLLK.A (2+) R.SQNAGAPVESYLAVQDK.T (3+) R.SQNAGAPVESYLAVQDK.T (2+)
30	XP_001597035	R.TNGAIGDGVTDDTAALQR.G (2+) R.TNGAIGDGVTDDTAALQR.G (3+) R.GINNAASSGQVFFLDAGTYK.V (2+) R.IVGESYSTIMSSGNFFNNINSPQPVVR.V (3+) R.IGGFTGSNLQVAQCEK.N (2+) R.IGGFTGSNLQVAQCEK.N (3+) R.QLDIYAGR.G (2+) R.NGQSQALYNANLNVYPTLITLYK.S (3+)
30	XP_001597035	K.ATPNFSGFGLIDADPYYSPVLNWGSTNVFLR.Q (3+) R.GINNAASSGQVFFLDAGTYK.V (3+) R.IVGESYSTIMSSGNFFNNINSPQPVVR.V (3+) R.NGQSQALYNANLNVYPTLITLYK.S (3+)

[a] Band number as given on the 1-D gel image.

[b] Accession number in NCBI (the same accession number in the different rows represent results from biological replicates 1 and 2, respectively)

[c] The matched peptides with the different charge generated by LC-MS/MS

Table S6-2. Detailed mass spectrometric data of proteins identified by 2-DE with LC-MS/MS in the sclerotial exudates

Spot [a]	Accession no. [b]	MS/MS Peptide sequence (charge) [c]
1	XP_001594660	K.EAFTFAWDGYQK.Y (2+) K.EAFTFAWDGYQK.Y (3+) K.YAFPHDELHPIANSYSDSR.N (3+) R.YVGGLLAGYDLLK.G (3+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) R.LFVSEHLACFDGGNFLLAGLVLK.E (3+) K.AGFYITNSDYILRPEVLESFYYAYR.V (3+) R.TGSGFAELENVNAPNGGGFNDFQDSFLFAEVMK.Y (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+) K.WVFNTEAHPLK.V (3+)
2	XP_001591483	R.SASDAFGLISIR.S (2+) R.SGSNLQNQAINAFQGGLWIGK.E (3+)
3	XP_001598009	K.QQDAFFGWNSVISTTNFEDK.G (2+) K.GPETVYAVGTFN.- (2+)
4	XP_001586352	K.TQADWENDFR.M (2+) R.MIQSFPGGFNSLR.V (2+) R.VYASSDCNTIANVVPAAIATGGK.V (3+) K.VLVGVWTEDAGHYDAEK.Q (3+) R.TYGFDWMVAVSVGSEDLYR.G (3+) R.GDTDAFTLSQQIYDVR.G (2+) R.GDTDAFTLSQQIYDVR.G (3+)
5	XP_001593425	R.VQSDFQAAFEAANSLVGTSGFTSAR.L (3+) R.VVGISVGSEDLYR.N (2+) K.SLFNAALAATQSATGK.T (2+)
6	XP_001588805	R.SDFFAFNDYSWCDPSSFTISGWDQK.V (3+) K.SLYSTDMTAVYSGGLVYEYSEEGSK.Y (3+) K.YGLVTINGGSVTEGPDFTALK.A (3+)
7	XP_001594660	K.EAFTFAWDGYQK.Y (2+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) R.LFVSEHLACFDGGNFLLAGLVLK.E (3+) K.AGFYITNSDYILRPEVLESFYYAYR.V (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+)
8	XP_001594660	K.EAFTFAWDGYQK.Y (2+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+)
9	XP_001588549	R.SINVLAIDHAAAGFNIGQR.A (3+) R.AMDTLTGGQAVALGR.I (2+)
10	XP_001588805	R.SDFFAFNDYSWCDPSSFTISGWDQK.V (3+) K.SLYSTDMTAVYSGGLVYEYSEEGSK.Y (3+) K.YGLVTINGGSVTEGPDFTALK.A (3+)
11	XP_001593909	K.CGTTPTLPVNGGASELPSPSGTLVYAALGR.G (3+) K.HFFDASGTPTFDLYFVNK.I (2+) K.HFFDASGTPTFDLYFVNK.I (3+) K.IGDVPAPEDADVGPANTGAVDWLALSAK.A (3+) K.SVGLSEVFR.V (2+) K.VGVESVPYAAQYWFYS.- (2+) K.VGVESVPYAAQYWFYS.- (3+)
12	XP_001586352	K.VLVGVWTEDAGHYDAEK.Q (3+) R.GDTDAFTLSQQIYDVR.G (3+)
13	XP_001586883	R.DQYTLSGTFPLIK.Y (2+) R.DQYTLSGTFPLIK.Y (3+) K.YEGIWPLDHSCGLTSTQGGR.Q (3+) R.QPMVAWESLTPAAQSALENTDFGK.A (3+)
14	XP_001588805	K.GNAFFIGDNR.F (2+) K.IVDPIADKDTCR.R (3+) R.SIPVGYSAADVDSNR.L (2+) R.SIPVGYSAADVDSNRLEMAQYMNCGTDDER.S (3+) R.LEMAQYMNCGTDDER.S (2+) R.LEMAQYMNCGTDDER.S (3+) R.SDFFAFNDYSWCDPSSFTISGWDQK.V (3+) K.SLYSTDMTAVYSGGLVYEYSEEGSK.Y (3+) K.YGLVTINGGSVTEGPDFTALK.A (2+) K.YGLVTINGGSVTEGPDFTALK.A (3+) K.AAFEGTTNPSGDGGYSSTNK.A (3+)
14	XP_001591030	K.SDLTTFMNNYAYFANK.A (2+) K.SDLTTFMNNYAYFANK.A (3+) R.GVSILVASGDSGVGTTCTTTGSTK.G (3+) R.GVSILVASGDSGVGTTCTTTGSTK.G (2+) K.AGLGFLNPWLYSTAK.S (2+) K.ITGCSGVISGAGFSAVSGWDPATGLGTPNYTK.L (3+)
15	XP_001591015	K.GITWGLYQEDMPYTGYQGMDWR.N (3+) R.NEVTGANAYVR.K (2+) R.KHNPEVLFTNVANKPER.L (3+) K.HNPEVLFTNVANKPER.L (3+) R.VFSILLGDAIPK.D (3+) R.VFSILLGDAIPK.D (2+) R.FDVGANVFSYVAQLTGDK.V (3+) R.FDVGANVFSYVAQLTGDK.V (2+) R.FDVGANVFSYVAQLTGDKVR.S (3+) R.SWTDGALESR.F (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (3+) K.DKDTIYQGQLEIPDGLNFEPNA.- (2+) K.DTIYQGQLEIPDGLNFEPNA.- (2+)
16	XP_001594345	R.ETVSATETLANTGAGNPVQEGTGLVR.S (3+) K.YGIVHDPIYGQVYAYEVDGFGSSNR.M (3+) R.ILTTSDPDEIYSTLK.E (2+) R.ILTTSDPDEIYSTLK.E (3+)
17	XP_001590395	R.AAGIDNYLTSESPIALQGVLNNIGPNGSK.A (3+) K.YLIDTANSSFQTLIEDYVTSQAK.L (3+) K.LQTVENPSGDLDSGAGLAEPK.F (2+) K.LQTVENPSGDLDSGAGLAEPK.F (3+) R.ATALISFGNDR.L (2+) R.SGLDCNSILTSIHTYDPDPAVGCDSTTFQPCSDR.A (3+) R.SIYTVNSDAPAGQAVAIGR.Y (3+) R.SIYTVNSDAPAGQAVAIGR.Y (2+) K.AMQDYADGFVAVAEK.Y (2+) K.AMQDYADGFVAVAEK.Y (3+) K.YTPEDGALAEQFSR.E (2+) K.YTPEDGALAEQFSR.E (3+)
18	XP_001594660	K.EAFTFAWDGYQK.Y (2+) K.YAFPHDELHPIANSYSDSR.N (3+) R.YVGGLLAGYDLLK.G (3+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) R.LFVSEHLACFDGGNFLLAGLVLK.E

Table S6-2. Continued.

		(3+) K.AGFYITNSDYILRPEVLESFYYAYR.V (3+) K.WVFNTEAHPLK.V (3+)
19	XP_001593430	K.GVTDCSGQVTETFDLGVK.T (2+) K.GVTDCSGQVTETFDLGVK.T (3+) K.TEGVESQGVCVGTGVMDGGK.W (3+) K.WYHGSGFLSCGC.- (2+)
20	XP_001595210	K.GGSELWTLLK.A (2+) R.VVRSQNAGAPVESYLAVQDK.T (3+) R.SQNAGAPVESYLAVQDK.T (3+) R.SQNAGAPVESYLAVQDK.T (2+)
21	XP_001593909	K.CGTTPTLPVNGGASELPSPSGTLVYAALGR.G (3+) K.HFFDASGTPTFDLYFVNK.I (2+) K.HFFDASGTPTFDLYFVNK.I (3+) K.IGDVPAPEDADVGPANTGAVDWLALSAK.A (3+) K.SVGLSEVFR.V (2+) K.VGVESVPYAAQYWFYS.- (2+) K.VGVESVPYAAQYWFYS.- (3+)
22	XP_001586883	R.DQYTLSGTFPLIK.Y (3+) R.DQYTLSGTFPLIK.Y (2+) K.YEGIWPLDHSCGLTSTQGGR.Q (2+) K.YEGIWPLDHSCGLTSTQGGR.Q (3+) K.NANFENNFVK.A (2+)
23	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) R.ILEVGTTFAQEDNGTWR.L (2+) R.ILEVGTTFAQEDNGTWR.L (3+) K.SSNSVLPDLVYIK.T (2+) K.SSNSVLPDLVYIK.T (3+) K.VEVHIASGASTYK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (3+) R.LLDVGSTFTQEQNGVWQLIDFNANGK.L (3+) K.YQNTGTGTVEVHVASG.- (2+)
24	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) R.ILEVGTTFAQEDNGTWR.L (3+) K.VEVHIASGASTYK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.YQNTGTGTVEVHVASG.- (2+) K.YQNTGTGTVEVHVASG.- (3+)
25	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) R.ILEVGTTFAQEDNGTWR.L (3+) K.SSNSVLPDLVYIK.T (3+) K.SSNSVLPDLVYIK.T (2+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (3+) R.LLDVGSTFTQEQNGVWQLIDFNANGK.L (3+) K.YQNTGTGTVEVHVASG.- (2+) K.YQNTGTGTVEVHVASG.- (3+)
26	XP_001586883	R.DQYTLSGTFPLIK.Y (2+) R.DQYTLSGTFPLIK.Y (3+) K.YEGIWPLDHSCGLTSTQGGR.Q (3+) R.QPMVAWESLTPAAQSALENTDFGK.A (3+)
27	XP_001593909	K.CGTTPTLPVNGGASELPSPSGTLVYAALGR.G (3+) K.HFFDASGTPTFDLYFVNK.I (3+) K.IGDVPAPEDADVGPANTGAVDWLALSAK.A (3+) K.SVGLSEVFR.V (2+) K.VGVESVPYAAQYWFYS.- (2+)
28	XP_001598370	R.LPFLFER.M (2+) R.MAQGSLTATLDATYLASYK.E (3+) K.EVVDYITAAGASAVLDAHNFGR.Y (3+) R.YNGNIITSTSDFGSFWSK.L (3+) R.YNGNIITSTSDFGSFWSK.L (2+) K.LIYEFHQYLDSDGSGTSATCVSSTIGAER.I (3+) R.IADATAWLK.A (2+)
29	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) K.VEVHIASGASTYK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (3+) K.YQNTGTGTVEVHVASG.- (3+)
30	XP_001598370	R.LPFLFER.M (2+) K.EVVDYITAAGASAVLDAHNFGR.Y (3+) R.YNGNIITSTSDFGSFWSK.L (3+) R.IADATAWLK.A (2+)
31	XP_001588805	K.GNAFFIGDNR.F (2+) R.SIPVGYSAADVDSNR.L (2+) R.SIPVGYSAADVDSNRLEMAQYMNCGTDDER.S (3+) R.LEMAQYMNCGTDDER.S (2+) R.SDFFAFNDYSWCDPSSFTISGWDQK.V (3+) K.SLYSTDMTAVYSGGLVYEYSEEGSK.Y (3+) K.YGLVTINGGSVTEGPDFTALK.A (3+) K.YGLVTINGGSVTEGPDFTALK.A (2+) K.AAFEGTTNPSGDGGYSSTNK.A (2+) K.AAFEGTTNPSGDGGYSSTNK.A (3+)
31	XP_001595540	R.SFVADTQSNGLVGLAFSK.L (2+) R.SFVADTQSNGLVGLAFSK.L (3+) K.TFFDNAMSQGLAMPVFTADLR.K (3+) K.ASAGSYEFGNIDSSK.F (2+) K.VQGAVNDQSVGGVTFPCSAK.L (2+) K.LPDLAVDVGGNYMAVVR.G (2+) K.LPDLAVDVGGNYMAVVR.G (3+) K.TCFGGLQATTSNLQIYGDIMFK.S (3+)
32	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) R.ILEVGTTFAQEDNGTWR.L (3+) K.VEVHIASGASTYK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+)
33	XP_001586339	R.EGWTSAGCYTDNVSGR.A (2+) R.EGWTSAGCYTDNVSGR.A (3+) K.TVDQNWVTAAQSAK.S (2+) K.SVLGFNEPDLTYEQSSNMLPEVAAQGYK.S (3+) R.IGGPGVLWNNWNSWSSGGGYSSR.V (3+) R.TVLPWLDSQGYVER.Y (2+) R.TVLPWLDSQGYVER.Y (3+)
34	XP_001591015	K.GITWGLYQEDMPYTGYQGMDWR.N (3+) R.VFSILLGDAIPK.D (2+) R.FDVGANVFSYVAQLTGDK.V (3+) R.FDVGANVFSYVAQLTGDK.V (2+) R.SWTDGALESR.F (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (3+) K.DTIYQGQLEIPDGLNFEPNA.- (3+)
35	XP_001591015	K.GITWGLYQEDMPYTGYQGMDWR.N (3+) R.NEVTGANAYVR.K (2+) R.KHNPEVLFTNVANKPER.L (3+) K.HNPEVLFTNVANKPER.L (3+) R.VFSILLGDAIPK.D (3+) R.VFSILLGDAIPK.D (2+) R.FDVGANVFSYVAQLTGDK.V (2+) R.FDVGANVFSYVAQLTGDK.V (3+) R.FDVGANVFSYVAQLTGDKVR.S (3+) R.SWTDGALESR.F (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (3+) K.DTIYQGQLEIPDGLNFEPNA.- (2+)
36	XP_001596398	K.FNSYLLTLQSSGDLGPNR.V (3+) K.YVMWMHIDSSNYGEAR.A (3+) R.AGVATSSSVCGSYTYLGASQPLGYQSR.D (3+) K.DTDGTGYLLTEDR.A (2+) K.SGAGNVIEFESYNSGWAPDIDR.I (3+)

Table S6-2. Continued.

37	XP_001591015	K.GITWGLYQEDMPYTGYQGMDWR.N (3+) R.VFSILLGDAIPK.D (2+) R.FDVGANVFSYVAQLTGDK.V (2+) R.FDVGANVFSYVAQLTGDK.V (3+) K.DKDTIYQGQLEIPDGLNFEPNA.- (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (3+) K.DTIYQGQLEIPDGLNFEPNA.- (3+)
37	XP_001591275	K.AGALAAVIYNNAHGALGGTLGAPDK.N (3+) K.HVDGSAYIDAVVQAIETTNIVAQTK.G (3+) R.LIAHSVATYAISFDGFPK.R (3+)
37	XP_001590904	R.FGIVTSVVLK.T (2+) K.SPIPLNVYFAWTSPSDDAYWR.G (3+) R.GLLQQTISHLTDVAK.S (2+)
38	XP_001591015	K.GITWGLYQEDMPYTGYQGMDWR.N (3+) R.VFSILLGDAIPK.D (2+) R.FDVGANVFSYVAQLTGDK.V (3+) R.FDVGANVFSYVAQLTGDK.V (2+) K.DKDTIYQGQLEIPDGLNFEPNA.- (3+)
39	XP_001594660	K.EAFTFAWDGYQK.Y (2+) R.YVGGLLAGYDLLK.G (2+) R.YVGGLLAGYDLLK.G (3+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+) K.WVFNTEAHPLK.V (3+)
40	XP_001594660	K.EAFTFAWDGYQK.Y (2+) R.YVGGLLAGYDLLK.G (2+) R.YVGGLLAGYDLLK.G (3+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+) K.WVFNTEAHPLK.V (3+)
41	XP_001590125	R.STFLMTVAGK.V (2+) R.QSVIGTYLYVSVK.S (2+) R.QSVIGTYLYVSVK.S (3+) R.QNQSEFNADYADDQAHWGYWLWSTK.A (3+) K.AVNGMSFQSGQDVVVR.G (2+) K.AVNGMSFQSGQDVVVR.G (3+) K.FAVDSLAAGGQDYLTITSLSAR.Q (2+) K.FAVDSLAAGGQDYLTITSLSAR.Q (3+) K.EISSDGNTQTVDVIFPAMPIFLYSNPVLLK.Y (3+) K.GIIAIEAMSR.I (2+) K.WETLAIVPATASVPAHTK.L (3+) K.LAYQDSTGHGLLYNLYADTLLK.L (3+) R.IYDMQSEYYPTIANAYGVQLDSR.N (3+) K.SDWEMWAAAIASPSTK.S (2+) K.LATWIGVTPTDR.A (2+)
42	XP_001594393	K.YGTVAQLPLVGTITNPLSGITVGR.A (3+) K.YGTVAQLPLVGTITNPLSGITVGR.A (2+) K.ILIDVSHVLPSFR.G (2+) K.ILIDVSHVLPSFR.G (3+) R.VGISWISK.E (2+) K.ACQNVNNEIPHDTIFSTVVSDTK.S (3+) K.SVWNNQVLSR.I (2+) R.CSTALFHVLQPTAYEEYIR.S (3+) R.SLIDIWR.H (2+) R.TQGGSNADNVLADAYVK.G (2+) R.GQINWNDGYAAMVK.D (2+) K.YGYITPTYSR.A (2+) R.AVDYAYNDFSLYQVASGLGK.T (2+) R.AVDYAYNDFSLYQVASGLGK.T (3+) K.KLTITSTGGDGNGDNDIYVQSLK.V (3+)
43	XP_001590125	R.QSVIGTYLYVSVK.S (2+) R.QNQSEFNADYADDQAHWGYWLWSTK.A (3+) K.FAVDSLAAGGQDYLTITSLSAR.Q (3+) K.GIIAIEAMSR.I (2+) K.LAYQDSTGHGLLYNLYADTLLK.L (3+) K.SDWEMWAAAIASPSTK.S (2+) K.LATWIGVTPTDR.A (2+)
43	XP_001594733	R.LEYISDVSDFSR.S (2+) R.IFLDALPEDAPYTVPYGTVAIDYLSLK.F (3+) K.CLDVFYPSVTDAGTTLIWK.G (3+) R.IGPVTYYSALYVLALENAAAIAK.S (3+) R.FYGNSFYDNDVLSDGFSQR.V (3+)
44	XP_001595304	K.GTPLIYEYTYHEMK.I (2+) K.GTPLIYEYTYHEMK.I (3+) K.IIVDGEFDISDETGQK.V (2+) K.IIVDGEFDISDETGQK.V (3+) K.GDVFYFPK.G (2+) K.ITFTTETFGLGFFVGQR.K (2+) K.ITFTTETFGLGFFVGQR.K (3+)
45	XP_001597818	R.SGVYEVTTQNSMDLGGSYDLAFK.G (3+) K.GSAVHGGGSCQVSITYDENPTK.D (3+) K.VIHSIIGGCPMR.N (3+) K.ATLAWTWFNK.I (2+)
46	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) K.NTGTGYVEVHIASSSSNFQTR.I (2+) R.ILEVGTTFAQEDNGTWR.L (2+) K.SSNSVLPDLVYIK.T (3+) K.SSNSVLPDLVYIK.T (2+) R.TVEVVTSFGNEQDGQWNVYDYDGDGKPDLVFIK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (3+) K.LDLTYIK.Y (2+) K.YQNTGTGTVEVHVASG.- (2+) K.YQNTGTGTVEVHVASG.- (3+)
47	XP_001584850	R.ILDTGTTFVEEHNGWWQLIDATGDGRPDLAYIK.N (3+) K.NTGTGYVEVHIASSSSNFQTR.I (3+) R.ILEVGTTFAQEDNGTWR.L (3+) R.ILEVGTTFAQEDNGTWR.L (2+) K.SSNSVLPDLVYIK.T (2+) K.SSNSVLPDLVYIK.T (3+) K.VEVHIASGASTYK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (3+) K.LDLTYIK.Y (2+) K.YQNTGTGTVEVHVASG.- (2+) K.YQNTGTGTVEVHVASG.- (3+)
48	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) R.ILEVGTTFAQEDNGTWR.L (3+) R.ILEVGTTFAQEDNGTWR.L (2+) K.SSNSVLPDLVYIK.T (3+) K.SSNSVLPDLVYIK.T (2+) R.TVEVVTSFGNEQDGQWNVYDYDGDGKPDLVFIK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (3+) R.LLDVGSTFTQEQNGVWQLIDFNANGK.L (3+) K.LDLTYIK.Y (2+) K.YQNTGTGTVEVHVASG.- (3+) K.YQNTGTGTVEVHVASG.- (2+)
49	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) K.SSNSVLPDLVYIK.T (2+) K.SSNSVLPDLVYIK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (3+)
50	XP_001593115	K.IILDVHGAPGSQNGFDNSGR.K (3+) K.QTLAAIQTLAYR.Y (2+) K.WTIVGEFSGAQTDCAK.W (3+) K.DVGTVDGLLAIDK.V (2+)
51	XP_001593115	K.IILDVHGAPGSQNGFDNSGR.K (3+) K.QTLAAIQTLAYR.Y (2+) R.YDGSYPGSPAVYGSCQTK.D (3+) K.DVGTVDGLLAIDK.V (2+)

Table S6-2. Continued.

52	XP_001596398	K.FNSYLLTLQSSGDLGPNR.V (2+) K.FNSYLLTLQSSGDLGPNR.V (3+) K.YVMWMHIDSSNYGEAR.A (3+) R.AGVATSSSVCGSYTYLGASQPLGYQSR.D (3+) K.DTDGTGYLLTEDR.A (2+) K.LSADYTSVVSAVYLFADYEAPAIYK.S (3+) R.WESSNLMTSTYVWLPLTISGTK.A (3+) R.VGYIGGPSPGGTLTIDGVSSSVATTTTIR.I (3+) R.YANVVVNGVSNIIAFLPTADGNTPGTSVLTTALK.S (3+) K.SGAGNVIEFESYNSGWAPDIDR.I (3+) K.SGAGNVIEFESYNSGWAPDIDR.I (2+)
53	XP_001597382	R.ADTFAITGVQDGGIQPR.L (2+) R.ADTFAITGVQDGGIQPR.L (3+) R.NGYPYSVYDTTLR.W (3+) R.NGYPYSVYDTTLR.W (2+) R.WPTSDTAAATSQNDQVESQLEANLR.N (3+) R.TQIMTLFANWQPYNHFSNK.G (3+) R.LLAIWQAVYPDSYVETAR.Q (3+) R.LLAIWQAVYPDSYVETAR.Q (2+) R.QGAATWTISR.G (2+) R.GSYQDQNSPLTPFHQDTNGK.F (3+) K.FFSPADVR.N (2+)
54	XP_001591947	R.QQYFTQLVGDAITLPTIK.G (3+) K.ATTDQIVYFDHGGYVVTDTIK.V (3+)
55	XP_001597382	R.ADTFAITGVQDGGIQPR.L (2+) R.ADTFAITGVQDGGIQPR.L (3+) R.NGYPYSVYDTTLR.W (2+) R.WPTSDTAAATSQNDQVESQLEANLR.N (3+) R.TQIMTLFANWQPYNHFSNK.G (3+)
56	XP_001591947	R.QGAPAYGDASYQIFR.N (2+) R.QGAPAYGDASYQIFR.N (3+) K.DFGAVGDGVTDDTAAINAAISAGNR.C (3+) K.INNCGIGLDMSAGGAASR.A (3+) R.AAGSATIMDSTITNTPIGISTVYDIK.E (3+) K.VYTGVNAGQATQGTQDVVTKPATLLDSTGK.L (3+) K.GDGVTDDTAAIQAIFDK.A (2+) K.ATTDQIVYFDHGGYVVTDTIK.V (3+)
56	XP_001595513	K.LGISNPGWWGISVQPQK.Y (2+) K.LGISNPGWWGISVQPQK.Y (3+) R.ASLGYPEPWK.I (2+) K.INYVEVGNEDNLSDGASSYHAYR.W (3+) K.SPFGPAFYVIGVNNPGEYVFK.A (3+) K.GTLTVLNAPDGNSMNVYGGEDVVVK.K (3+)
57	XP_001590125	R.QSVIGTYLYVSVK.S (2+) K.AVNGMSFQSGQDVVVR.G (3+) K.GIIAIEAMSR.I (2+) K.WETLAIVPATASVPAHTK.L (3+) K.LAYQDSTGHGLLYNLYADTLLK.L (3+) R.IYDMQSEYYPTIANAYGVQLDSR.N (3+) K.SDWEMWAAAIASPSTK.S (2+) K.SDWEMWAAAIASPSTK.S (3+) K.LATWIGVTPTDR.A (2+)
58	XP_001596362	K.NIDLEILVK.N (2+) K.SSQIFNVGTNSIVK.E (2+) K.SSQIFNVGTNSIVK.E (3+) K.ELPELAGDYR.T (2+) R.IQPLSANPTWELDSMPEGR.G (3+) K.APTLEALLYDPTK.A (2+) R.FSTLATSTIPR.L (2+) K.LKPDAADEFVTEFR.V (3+) R.VENYVPPYLSGDNANK.R (2+) K.ADGSTLDVTFNCPAGAK.A (3+) K.ADGSTLDVTFNCPAGAK.A (2+) R.MLHLDNTGFVAGATQQK.L (3+)
5701	XP_001590125	R.QSVIGTYLYVSVK.S (2+) K.FAVDSLAAGGQDYLTITSLSAR.Q (3+) R.QAFGAVQLCGTPDKPYYFLK.E (3+) K.WETLAIVPATASVPAHTK.L (3+) R.IYDMQSEYYPTIANAYGVQLDSR.N (3+) K.SDWEMWAAAIASPSTK.S (2+) K.LATWIGVTPTDR.A (2+)
59	XP_001595210	K.GGSELWTLLK.A (2+) R.VVRSQNAGAPVESYLAVQDK.T (3+) R.SQNAGAPVESYLAVQDK.T (3+) R.SQNAGAPVESYLAVQDK.T (2+)
60	XP_001589214	R.YDQGCLLLHLTK.A (3+) K.DVEVTIEVR.R (2+) K.SLWVYWVR.G (2+) K.FWDFALEQR.A (2+)
61	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) K.NTGTGYVEVHIASSSSNFQTR.I (2+) R.ILEVGTTFAQEDNGTWR.L (3+) R.ILEVGTTFAQEDNGTWR.L (2+) K.SSNSVLPDLVYIK.T (2+) K.SSNSVLPDLVYIK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (2+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) R.LISTGTTFTVENNGFWQLGPYSANGDLIYIK.D (3+) K.DANTGTGTTEVHIASR.A (2+) K.DANTGTGTTEVHIASR.A (3+) R.LLDVGSTFTQEQNGVWQLIDFNANGK.L (3+) K.LDLTYIK.Y (2+) K.YQNTGTGTVEVHVASG.- (2+) K.YQNTGTGTVEVHVASG.- (3+)
62	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) R.ILEVGTTFAQEDNGTWR.L (2+) R.ILEVGTTFAQEDNGTWR.L (3+) K.SSNSVLPDLVYIK.T (2+) K.SSNSVLPDLVYIK.T (3+) K.VEVHIASGASTYK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (3+) R.LLDVGSTFTQEQNGVWQLIDFNANGK.L (3+) K.LDLTYIK.Y (2+) K.YQNTGTGTVEVHVASG.- (2+) K.YQNTGTGTVEVHVASG.- (3+)
63	XP_001584850	K.NTGTGYVEVHIASSSSNFQTR.I (3+) K.SSNSVLPDLVYIK.T (2+) K.VEVHIASGASTYK.T (3+) K.TSNTGTGTTELFVASASSNYQTR.L (3+) K.DANTGTGTTEVHIASR.A (3+)
64	XP_001595093	R.SLSGYSWDISYADGSGASGVVGTDTVTIGK.T (3+) K.YTGSLTYTSVSSGNGFWEFPSTSYK.V (3+) K.GASYSNSYGGYVFPCSATLPTLSFK.I (3+)
65	XP_001586339	K.YGDASFGNMFR.Q (2+) K.TVDQNWVTAAQSAK.S (2+) K.SWIQPFAGQVR.I (2+) R.IGGPGVLWNNWNSWSSGGGYSSR.V (3+) R.TVLPWLDSQGYVER.Y (2+) R.TVLPWLDSQGYVER.Y (3+)
66	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (3+) K.DPWHTETILEEK.W (3+) K.KEEELINFFPGSK.L (3+) R.NLNPVNEVGTLENENGSWK.H (3+)
67	XP_001597382	R.NGYPYSVYDTTLR.W (2+) R.NGYPYSVYDTTLR.W (3+) R.WPTSDTAAATSQNDQVESQLEANLR.N (3+)

Table S6-2. Continued.

		R.TQIMTLFANWQPYNHFSNK.G (3+) R.LLAIWQAVYPDSYVETAR.Q (3+)
68	XP_001593115	K.IILDVHGAPGSQNGFDNSGR.K (3+) K.QTLAAIQTLAYR.Y (2+) R.YAPATDVVTGIELLNEPANWALDMGAVK.Q (3+) K.QFYYDGWGNVR.N (2+) K.WTIVGEFSGAQTDCAK.W (2+) K.DVGTVDGLLAIDK.V (2+)
69	XP_001597382	R.ADTFAITGVQDGGIQPR.L (2+) R.ADTFAITGVQDGGIQPR.L (3+) R.NGYPYSVYDTTLR.W (3+) R.NGYPYSVYDTTLR.W (2+) R.WPTSDTAAATSQNDQVESQLEANLR.N (3+) R.LLAIWQAVYPDSYVETAR.Q (2+) R.LLAIWQAVYPDSYVETAR.Q (3+) R.GSYQDQNSPLTPFHQDTNGK.F (3+)
70	XP_001597382	R.ADTFAITGVQDGGIQPR.L (3+) R.LLAIWQAVYPDSYVETAR.Q (3+)
71	XP_001597382	R.ADTFAITGVQDGGIQPR.L (2+) R.ADTFAITGVQDGGIQPR.L (3+) R.NGYPYSVYDTTLR.W (2+) R.WPTSDTAAATSQNDQVESQLEANLR.N (3+) R.LLAIWQAVYPDSYVETAR.Q (3+) R.LLAIWQAVYPDSYVETAR.Q (2+) R.GSYQDQNSPLTPFHQDTNGK.F (3+)
72	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.NIDLEILVK.N (2+) K.APTLEALLYDPTK.A (2+)
73	XP_001594660	K.EAFTFAWDGYQK.Y (2+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+)
74	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.NIDLEILVK.N (2+) K.APTLEALLYDPTK.A (2+) K.LKPDAADEFVTEFR.V (3+)
75	XP_001589422	R.VLSITTPGSTVYSGDR.E (2+) R.LLNEWQAALVR.T (2+) R.YQVAHVGDSFPSVIDR.F (3+) R.FWWDTAGPTFPHQVLGLVAYDVPK.T (3+) K.TQLTFGTDWPFR.R (2+)
76	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.NIDLEILVK.N (2+) R.GMVEGNLLPDGTVIWLNGGNLGAQGFGLAK.A (3+) R.MLHLDNTGFVAGATQQK.L (3+)
76	XP_001584844	K.NGAVASESSICSQIGIDIMK.E (2+) K.NGAVASESSICSQIGIDIMK.E (3+)
77	XP_001590125	R.QSVIGTYLYVSVK.S (2+) R.QSVIGTYLYVSVK.S (3+) R.QNQSEFNADYADDQAHWGYWLWSTK.A (3+) K.FAVDSLAAGGQDYLTITSLSAR.Q (3+) R.QAFGAVQLCGTPDKPYYFLK.E (3+) K.EISSDGNTQTVDVIFPAMPIFLYSNPVLLK.Y (3+) K.LAYQDSTGHGLLYNLYADTLLK.L (3+) R.IYDMQSEYYPTIANAYGVQLDSR.N (3+) K.SDWEMWAAAIASPSTK.S (2+)
77	XP_001593684	K.NVILINDQFPGPLIEANWGDVINVR.V (3+) K.LSPWADGVPSVSQCPIAPGSSFTYEFR.A (3+) R.ANYNSPLLLLASEGTPLAPLPK.D (3+)
78	XP_001590125	R.STFLMTVAGK.V (2+) R.QSVIGTYLYVSVK.S (2+) R.QNQSEFNADYADDQAHWGYWLWSTK.A (3+) K.AVNGMSFQSGQDVVVR.G (2+) K.AVNGMSFQSGQDVVVR.G (3+) K.FAVDSLAAGGQDYLTITSLSAR.Q (2+) R.QAFGAVQLCGTPDKPYYFLK.E (3+) K.EISSDGNTQTVDVIFPAMPIFLYSNPVLLK.Y (3+) K.GIIAIEAMSR.I (2+) K.WETLAIVPATASVPAHTK.L (3+) K.LAYQDSTGHGLLYNLYADTLLK.L (3+) R.IYDMQSEYYPTIANAYGVQLDSR.N (3+) K.SDWEMWAAAIASPSTK.S (2+) K.SDWEMWAAAIASPSTK.S (3+) K.LATWIGVTPTDR.A (2+)
78	XP_001593684	K.LSPWADGVPSVSQCPIAPGSSFTYEFR.A (3+) K.VPNGPVFPNVDNNLINGK.G (3+) R.ANYNSPLLLLASEGTPLAPLPK.D (3+) R.GTWDGSITNPSNPLR.R (2+)
79	XP_001596362	R.LPNGNYAMSSEYDPATNGVAAPLAYK.T (3+) R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.NIDLEILVK.N (2+) K.SSQIFNVGTNSIVK.E (2+) K.ELPELAGDYR.T (2+) R.IQPLSANPTWELDSMPEGR.G (2+) R.IQPLSANPTWELDSMPEGR.G (3+) K.APTLEALLYDPTK.A (2+) K.APTLEALLYDPTK.A (3+) R.FSTLATSTIPR.L (2+) K.LKPDAADEFVTEFR.V (2+) R.VENYVPPYLSGDNANK.R (2+) R.VENYVPPYLSGDNANK.R (3+) K.ADGSTLDVTFNCPAGAK.A (2+) K.ADGSTLDVTFNCPAGAK.A (3+) R.MLHLDNTGFVAGATQQK.L (3+)
80	XP_001596362	R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.SSQIFNVGTNSIVK.E (2+) K.SSQIFNVGTNSIVK.E (3+) K.ELPELAGDYR.T (2+) R.IQPLSANPTWELDSMPEGR.G (3+) K.APTLEALLYDPTK.A (2+) R.FSTLATSTIPR.L (2+) R.VENYVPPYLSGDNANK.R (3+) K.ADGSTLDVTFNCPAGAK.A (2+) K.ADGSTLDVTFNCPAGAK.A (3+) R.MLHLDNTGFVAGATQQK.L (3+)
81	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) R.SPDGGNVWTTQDTIIAK.D (3+) K.DGDYNGSSMTCYYVDK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.DPWHTETILEEK.W (3+) K.WGDELPGTALACVIGNGK.I (2+) K.WGDELPGTALACVIGNGK.I (3+) K.DGSVHLFYVSK.S (2+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (2+) R.NLNPVNEVGTLENENGSWK.H (2+) R.NLNPVNEVGTLENENGSWK.H (3+)
82	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (3+) K.DANFDNQPSIHLLYMNK.D (3+) K.LTSGSFNQGTGWNPNGSQWAYFSASK.D (3+) K.DPWHTETILEEK.W (3+) K.WGDELPGTALACVIGNGK.I (3+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (2+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (3+)
83	XP_001597582	K.SSCSDTTLLGSFLENHDVAR.F (3+) R.NQAIYVDPTYLTYK.A (2+) K.SYLVGSGVCSL.- (2+)
84	XP_001596362	R.LPNGNYAMSSEYDPATNGVAAPLAYK.T (3+) R.VVSLGGNAPLSWLDPTIGDGFTAIR.Y (3+) K.NIDLEILVK.N (2+) K.SSQIFNVGTNSIVK.E (2+) K.ELPELAGDYR.T (2+) R.IQPLSANPTWELDSMPEGR.G (3+) K.APTLEALLYDPTK.A (2+)

Table S5-2. Continued.

		R.FSTLATSTIPR.L (2+) K.LKPDAADEFVTEFR.V (3+) R.VENYVPPYLSGDNANK.R (3+) K.ADGSTLDVTFNCPAGAK.A (2+) K.ADGSTLDVTFNCPAGAK.A (3+) R.MLHLDNTGFVAGATQQK.L (3+)
84	XP_001596243	K.YATTSLTSGPGLTVGSSISLR.A (3+) R.HYNFGLLLNANDGSK.Q (3+)
85	XP_001589422	R.VLSITTPGSTVYSGDR.E (3+) R.LLNEWQAALVR.T (3+) R.LLNEWQAALVR.T (2+) R.YNGVLIEANPTIYPPR.Q (3+) R.QVEFYFDTAR.T (2+) R.YQVAHVGDSFPSVIDR.F (3+) K.TQLTFGTDWPFR.R (2+) R.RLYDIGPL.- (2+)
86	XP_001596243	K.AYGVFISPGTGYR.N (2+) R.GANSASGSLSTYYSGVRPDASGYNPMSK.E (3+) K.YATTSLTSGPGLTVGSSISLR.A (2+) K.YATTSLTSGPGLTVGSSISLR.A 93+) R.YIAHTGSTVNTQVVTTSSATALK.Q (3+) R.TGLGNSACFSFESNDTPGSYIR.H (3+) R.HYNFGLLLNANDGSK.Q (3+)
87	XP_001594397	R.AFIVGDVNNPSNR.F (2+) R.FIYLVLDTASGDTAIR.Y (2+) R.FIYLVLDTASGDTAIR.Y (3+) K.QSYQAIVDGAVLSIK.R (2+) K.QSYQAIVDGAVLSIK.R (3+) R.SLYAYLANPAAER.A (3+) R.SLYAYLANPAAER.A (2+) K.GVAAYLFEK.A (2+) R.GPAFQALDLGVTSCYIIGQR.Q (3+) R.QYDAAQALYNSLDSVGTPLVDGSVK.S (3+) R.VGQLIIIISPSEATTMSGR.R (3+) K.VVLGGPANTYAHYLATPEEYTVQR.Y (3+)
88	XP_001594397	R.FIYLVLDTASGDTAIR.Y (2+) K.QSYQAIVDGAVLSIK.R (2+) R.SLYAYLANPAAER.A (2+) R.SLYAYLANPAAER.A (3+) K.GVAAYLFEK.A (2+) R.GPAFQALDLGVTSCYIIGQR.Q (3+) R.QYDAAQALYNSLDSVGTPLVDGSVK.S (3+) R.VGQLIIIISPSEATTMSGR.R (3+) K.VVLGGPANTYAHYLATPEEYTVQR.Y (3+)
89	XP_001594278	R.TTYNFFENSSGFGTTSVDVR.V (3+) R.VTGLSGSTVIIK.N (2+) K.NVAVTPGLVVTASGNLK.S (2+)
90	XP_001584968	R.SPDGGNVWTTQDTIIAK.D (2+) R.SPDGGNVWTTQDTIIAK.D (3+) K.DGDYNGSSMTCYYVDK.D (2+) K.DANFDNQPSIHLLYMNK.D (3+) K.DANFDNQPSIHLLYMNK.D (2+) K.GDNPIWEDAKVPDEVR.K (3+) K.DPWHTETILEEK.W (2+) K.DPWHTETILEEK.W (3+) K.WGDELPGTALACVIGNGK.I (2+) K.WGDELPGTALACVIGNGK.I (3+) R.VFLQHHDYSIILYENQNNTWHDR.G (3+) K.DGSVHLFYVSK.S (2+) K.DGSVHLFYVSK.S (3+) K.KEEELINFFPGSK.L (2+) K.KEEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (3+) K.EEELINFFPGSK.L (2+) K.ITLFFR.N (2+) R.NLNPVNEVGTLENENGSWK.H (2+) R.NLNPVNEVGTLENENGSWK.H (3+)
91	XP_001597228	K.TATCEAYVTANPSAFK.N (2+) K.TATCEAYVTANPSAFK.N (3+) K.NAYWIINSIK.V (2+)
92	XP_001594660	K.EAFTFAWDGYQK.Y (2+) R.YVGGLLAGYDLLK.G (2+) R.LADNLAFAFDTPTGVPSNNVYLNPPR.T (3+) R.LFVSEHLACFDGGNFLLAGLVLK.E (3+) K.AGFYITNSDYILRPEVLESFYYAYR.V (3+) K.YAYLIHGDDEEYQVGGGGVNK.W (3+)

[a] Respective identification number of spots as given on the 2-D gel image

[b] Accession number in NCBI database

[c] The matched peptides with the different charge generated by LC-MS/MS