Metabolomic changes of Brassica rapa under biotic stress

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Chapter 2

The role of secondary metabolites in *Arabidopsis* and *Brassica* in the interaction with fungi

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Abstract

Brassica is a very important plant genus, not only as a source of oil and protein for human and animal nutrition, but also as a potential source of allelochemical control for a variety of soil-borne pests. The discovery of anticarcinogenic effects of *Brassica*'s secondary metabolites has increased the importance of this genus. *Brassica* also plays a very important role in ecosystems. *Arabidopsis* is one of the most important model plants suitable to study the interaction between plant and pathogens. Availability of numerous collections and mutants help in using this plant in developing novel crop protection strategies.The objective of this paper is to review the interaction between *Brassica* and *Arabidopsis* plants with pathogenic fungi and to explain the role of glucosinolates, phytoalexins and other secondary metabolites in defense against these detrimental pathogenic fungi. The advent of new technologies for the analysis of gene expression combined with the recent progress in exploring the complete genome sequence in many plants have led to a good understanding of the plant-fungi interactions. Metabolite measurements, metabolomic profiling and multivariate analysis are considered as promising approaches for evaluating particular signaling pathways in defense responses.

Keywords: *Alternaria brassicicola, Arabidopsis*, *Brassicas*, glucosinolates, *Leptosphaeria maculans*, phytoalexins, pathogenic fungi, plant resistance mechanism

2.1 Introduction

The genus *Brassica* is an important source of vegetable oils (Sasaki and Takahshi, 2002; Abramson and Smith, 2003), proteins for human (Font *et al*., 2005) and animal nutrition (Slominski *et al*., 1999; Font *et al*., 2005). The recent popularity of *Brassica* has resulted in a considerable increase in its production in Asia, Europe and the USA not only as a source of vegetables but also because of the edible seeds and industrial oils extracted from the seeds (Sasaki and Takahshi, 2002). The species *Brassica rapa* includes many significant crops, such as Chinese cabbage, which is common in China, Japan, and other Asian countries. *Brassica rapa* ssp. *pekinensis* and spp. *rapa* can be dried, pickled, or cooked for daily food (Li, 1962; Tan, 1979; Lim *et al*., 2001; Padilla *et al*., 2007). Because of its great and diverse economical interests, knowledge of the phytochemistry of Brassicaceae is of great importance. The phytochemical composition of plants depends on its genetic background and environmental factors. Knowledge of the natural products involved in resistance has not only importance for a better understanding of resistance, but also because of health affecting properties of various Brassicaceae vegetables and oils.

A major goal of plant science is the production of crops with increased and durable resistance to the spectrum of diseases. In the past, such resistance has been sought through traditional breeding approaches or by the widespread application of pesticides (Gurr and Rushton, 2005). As genetic engineering offers novel perspectives of improving natural resistance in a more targeted and faster fashion than traditional breeding, now there is an increased interest to understand the natural resistance. Even traditional breeding can be more effective by knowing the genes involved in resistance (Gurr and Rushton, 2005). Moreover, plants do affect the rhizosphere and suppress pathogenic fungi. In this respect Brassicaceae plants represent a potential source of allelochemical control for a variety of soil-borne pests (Brown and Morra, 1997; Bending and Lincoln, 1999; Morra and Kirkegaard, 2002).

In recent years, some attention has been paid to health improving effects of *Brassica* vegetables in our diet. The anticarcinogenic effect of Brassicaceae has been attributed to the effect of flavonoids, phenylpropanoids, glucosinolates, and the byproducts of the hydrolysis of the aliphatic and aromatic glucosinolates (Talalay and Fahey, 2001; Branca *et al*., 2002; Vallejo *et al*., 2004a,b). Natural isothiocyanates derived from the aliphatic and aromatic glucosinolates are effective chemoprotective

agents that block chemical carcinogenesis and prevent several types of cancer in rodent models (Wattenberg, 1992; Mithen, 2001). The isolated isothiocyanate, sulforaphane was shown to inhibit 9,10-dimethyl-1,2-benzanthracene-induced mammary tumors, when administered 3h before the carcinogen. These naturally occurring compounds can inhibit carcinogenesis and have a special role in cancer prevention (Heaney and Fedwick, 1993; Hecht, 2000). Sulforaphane has been shown to reduce the incidence of a number of tumor forms in various experimental models, both *in vivo* in animals and *in vitro* in cell cultures (Zhang *et al*., 1994). Consumption of cruciferous vegetables such as broccoli, Brussels sprouts and cabbage results in lower probability of acquiring colon and rectal cancers (Kohlmeier and Su, 1997), pancreatic cancer (Olsen *et al*., 1989), lung cancer (Le Marchand *et al*., 1989), reduction of incidence of cancer to the bladder (Michaud *et al*., 1999), and prostate (Cohen *et al*., 2000). The wild ecotype of *Arabidopsis thaliana* is a rich source of methylsulfinylalkyl which has the ability to inhibit metabolic activation of carcinogens and or by inducing carcinogen-detoxifying enzymes (Gross *et al*., 2000).

Also flavonoids have biological activities against various types of cancer and cardiovascular diseases. Broccoli inflorescences have been shown to be good sources of the health promoting compounds such as flavonoids, hydroxycinnamic acids, and vitamins (Vallejo *et al*., 2004a). Natural phenylpropanoids such as caffeoyl-1-malic acid have an important role in protection of the endothelial cells against oxidized low density lipoproteins-induced cytotoxicity (Martin-Nizard *et al*., 2003).

Because *A. thaliana* has become a model system for plant biochemistry, this plant now enables to study many general aspects of plant biology and biochemistry. The function of this plant's secondary metabolites can be studied due to the availability of numerous mutants, mapping tools and the full genomic sequence of this plant. It provides an excellent model among others for studying the formation and function of glucosinolates and phenylpropanoids (Reichelt *et al*., 2002). It also will be of great value to apply the results to closely related plant species of the same family.
Here we will review the phytochemical aspects of Brassicaceae in connection to their interaction with microorganisms.

2.1.1 Function of secondary metabolites

Secondary metabolites, at least the major ones present in a plant, apparently function as defense against herbivores, microbes, and viruses (Gardner, 1977; Wink,

2003; Wink and Mohamed, 2003). Secondary metabolites from Leguminosae (alkaloids, amines, flavonoids, isoflavonoids, coumarins, phenylpropanoids, anthraquinones, di-, sesqui- and triterpenes) and from Brassicaceae (phenylpropanoids, flavonoids and glucosinolates) function as defense chemicals against a wide variety of microorganisms and herbivores (Wink, 1988 and 2003; Birch *et al*., 1992; Parvez *et al*., 2004). In addition to the role of secondary metabolites in plant defense mechanism, some of them have physiological functions, for example they can serve as mobile and toxic nitrogen transport and storage compounds or UV-protectants. These multiple functions do not contradict the main role of secondary metabolites as chemical defense and signal compounds (Wink, 2003).

2.1.2 Constitutive versus inducible defense in plants

Mechanical barriers such as thick cuticles, thorns and hairs are the first defense barrier. The next barrier is chemical, e.g. polygalacturonase-inhibiting proteins, pectic oligomers and exudate compounds, which encounter the fungi during their infection and inhibit spore germination and germ tube elongation. These barriers are considered to be part of the arsenal of constitutive or preformed antifungal compounds produced by plants. These antifungal compounds are also known as preinfectional metabolites, prohibitins, or phytoanticipins (Grayer and Kokubun, 2001). Higher plants have the ability to initiate various defense reactions such as the hypersensitive response (HR) and systemic acquired resistance (SAR) (Ryals *et al*., 1994). In many incompatible plant-pathogen interactions, the pathogen induces the earliest defense response, a hypersensitive reaction and the activation of a series of defense responses including production of antimicrobial compounds (phytoalexins), oxidants, cell wall reinforcement and lytic enzymes in the infected and surrounding cells. HR by the rapid appearance of necrotic lesions, contributes to limitation of pathogen spread and is often correlated with systemic acquired resistance against subsequent pathogen attack (Petitot *et al*., 1997). SAR results in a coordinated activation of several families of SAR genes especially genes encoding pathogenesis-related (PR) proteins correlated with the development of resistance (Ryals et al., 1994; Suty *et al*., 2003). A local oxidative burst along with a hypersensitive response characterized by programmed cell death (PCD), leads to necrosis at the site of infection and induces a plant-wide resistance against further attack by a variety of

microbial pathogens. This phenomenon is known as induced systemic resistance (ISR) (Heil, 2001). Induced systemic resistance against pathogen infection has been described long time ago. Over the past decades, research on the induced systemic resistance against microbial pathogens has focused on specificity of interactions, signaling pathways and how these findings might be used in crop production and breeding programs (Ryals *et al*., 1994; Heil, 2001). The researchers concluded that the specificity of the induced resistance was not directly dependent on the type of inducing pathogen. Also the local infection by single strains of bacteria, viruses and fungi induces many plant species to synthesize signal molecules (such as salicylic and jasmonic acids) at the site of infection which consequently spread systemically throughout the whole plant (Ryals *et al*., 1994; Heil, 2001).

2.2 Overview of phytochemistry

Plants attacked by pathogenic fungi can produce antifungal secondary metabolites. Phytoalexins (antimicrobial compounds formed in plants via a metabolic sequence induced either biotically or abiotically as response to chemical or environmental factors) (Bailey and Mansfield, 1982), including flavonoids, terpenes, phenylpropanoids, phenolics, iridoid glucosides, glucosinolates and saponins. The structures are often unique at the plant family level or even species specific (Grayer and Kokubun, 2001; Brooks and Watson, 1985; Conn *et al*., 1988; Dahiya and Rimmer, 1988; Olsson *et al*., 1998; Pedras *et al*., 2002). A large number of biological and chemical studies have focused on the secondary metabolites of plants of economic importance, for example, metabolites of the plant families Leguminosae and Brassicaceae (Grayer and Kokubun, 2001; Pedras *et al*., 2002; Bennett and Wallsgrove, 1994).

Phenylpropanoids, sesquiterpenes, flavonoids and glucosinolates are the main and most important secondary metabolites produced in Brassicaceae especially after elicitation with biotic or abiotic factors. All known phytoalexins produced by members of Brassicaceae have a common chemical structure. These compounds are sulfur–containing indoles derivatized at the C-3 position of the indole ring (Devys *et al*., 1990; Hammerschmidt *et al*., 1993; Pedras *et al*., 1997a) (**Figure 2.1A**). Phenylpropanoids are a class of plant-derived organic compounds that are biosynthesized from the amino acid phenylalanine.

They have a wide variety of functions, including defense against herbivores, microbial attack, and other sources of injury. Flavonoids are polyphenolic antioxidants naturally present in vegetables and fruits. Flavonoids inhibit oxidation of low-density lipoprotein and reduce thrombotic tendency (Hertog *et al*., 1993).

Glucosinolates are a group of secondary metabolites found in all species of the family Brassicaceae. The glucosinolates contain a β-D-glucopyranosyl unit, a cyano group, and a sulfate group. They are classified into three different classes depending on the precursor amino acids, from which they are derived: aliphatic glucosinolates derived from methionine, aromatic glucosinolates derived from phenylalanine and tyrosine, and indole glucosinolates derived from tryptophan (Reichelt *et al*., 2002; Stoewsand, 1995; Halkier and Du, 1997; Kiddle *et al*., 2001; Brown *et al*., 2003).

2.3 Phytochemistry of Brassicaceae

2.3.1 Phenylpropanoids and flavonoids

Although, several investigations were performed to study the role of phenylpropanoids in defense in many plant species, there are few reports about phenylpropanoids in Brassicaceae. By using HPLC, MS, and NMR analyses of the cell wall extracts from *Arabidopsis* roots, eleven aromatic compounds have been identified. Nine of them together constituted three series of 4-hydroxy-, 4-methoxy, and 4-hydroxy-3,5-dimethoxy-substituted benzaldehyde, benzoic acid and cinnamic acid. The other two were indole dervatives (indole-3-carboxylic acid and indole-3-carbaldehyde). Several of these compounds, indole-3-carboxylic, 4-hydroxy benzoic acid and the four aldehydes increase considerably in concentration as a result of infection of *Arabidopsis* roots with *Pythium sylvaticum.* The same pattern of variation of aromatic compounds has been noticed in the leaves of *Arabidopsis* after infection with *Pseudomonas syringae* (Tan *et al*., 2004).

The metabolomic profiling of *B. rapa* leaves after treatment with MJ was studied. Ten phenylpropanoids (*trans* and *cis* forms) were identified using one and two dimensional NMR spectroscopy and HPLC-MS. Sinapoyl-, coumaroyl-, feruloyl- and caffeoyl malate were elucidated with new phenylpropanoids (5-hydroxyferuloyl malates) from leaves (Liang *et al*., 2006a).

Flavonoid profiling has been used for clarifying taxonomic relationships among the cruciferous plants. The leaves of cruciferous plants accumulate flavonol derivatives (quercetin, kaempferol and isorhamnetin) and flavone derivatives (apigenin and luteolin) (Onyilagha *et al*., 2003) (**Figure 2.1D**).

Brassica napus accumulates the three flavonol derivatives (quercetin, kaempferol, and isorhamnetin) (Onyilagha *et al*., 2003).

By using liquid chromatography-UV diode-array detection-electrospray ionization mass spectroscopy, the composition of broccoli inflorescences and characterization of a large number of hydroxycinnamic acids esters of kaempferol, quercetin and isorhamnetin glucosides were carried out. These glucosides were identified as 3-sophoroside of kaempferol, sophorotrioside-7-glucoside of quercetin and isorhamnetin sophoroside in addition to other less complex glucosides (Vallejo *et al*., 2004). Isorhamnetin 3,7-*O*-di-β-D-glucopyranoide was separated and identified from the corolla of *B. rapa* flowers (Lim *et al*., 2001). From *B. alba*, three flavonoids from shoots, roots and root exudates were isolated and identified: 3,5,6,7,8-pentahydroxy-4′-methoxy flavone, 2′,3′,4′,5′,6′-pentahydroxy chalcone and 3,5,6,7,8-pentahydroxy flavone and for the first time a flavonoid (apigenin) was elucidated from the shoots and roots (Ponce *et al*., 2004).

Researchers have used both biotic and abiotic elicitors to monitor the inducible secondary metabolites in plants. Kaempferol 3-*O*-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside, kaempferol 3,7-*O*-α-L-dirhamnopyranoside and quercetin glycosides were identified in methyl jasmonate treated *Arabidopsis* using two dimensional nuclear magnetic resonance and principal component analysis (PCA) (Hendrawati *et al*., 2006). UV-B radiation was used in two cultivars of *B. napus* to enhance the overall amounts of soluble flavonoids. Quercetin glucosides and kaempferol increased by 150% in the atrazin sensitive cultivar compared to control plants and increased by 70% in the atrazin tolerant cultivar compared to control plants from *B. napus* (Olsson *et al*., 1998).

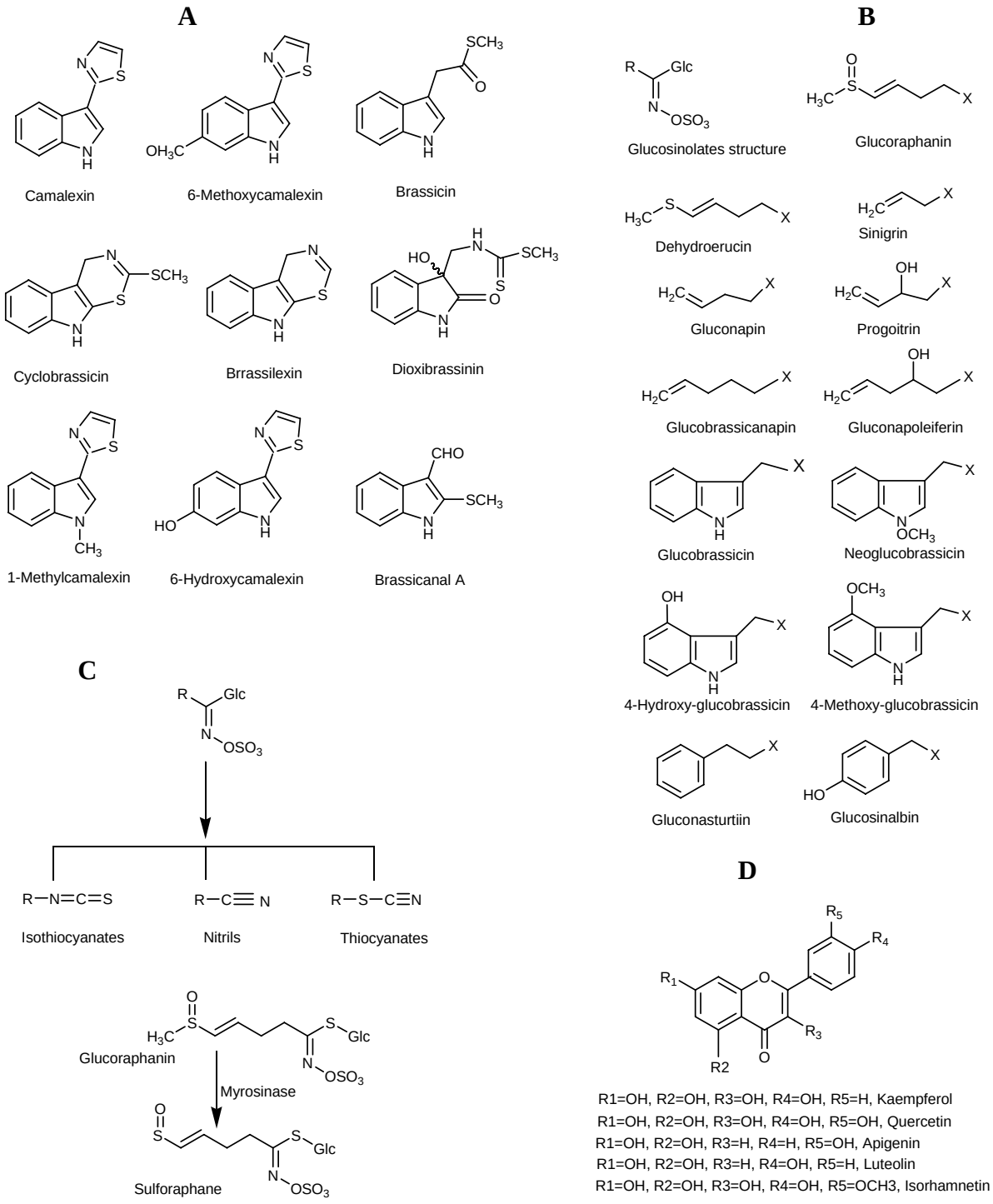

Figure 2.1. Chemical structures of some antifungal secondary metabolites in Brassicaceae, **A**: Phytoalexins, **B**: Glucosinolates, **C**: Glucosinolates hydrolysis by myrosinase enzyme, **D**: Flavonoids.

Four of the flavonol glucosides were identified after UV-B radiation: quercetin- and kaempferol 3-sophoroside-7-glucoside and 3-(2′-E-sinapoylsophoroside)-7-glucoside, respectively (Olsson *et al*., 1998).

In spite of the importance of phenylpropanoids and flavonoids as plant secondary metabolites in plant resistance mechanisms and anticarcinogenic effects, few studies have been done in Brassicaceae. This is clear from the few publications about the phenylpropanoids and flavonoids in Brassicaceae. Identification of phenylpropanoids and flavonoids in *Brassica* before and after infection with pathogens may throw new light on the plant's resistance mechanisms.

2.3.2 Terpenes

Monoterpenes (sabinene, limonene, α-thujene, 1,8-cineole, β-pinene, myrcene, α-pinene and γ–terpinene) were emitted from cabbage cv. Lennox grown at high CO_2 concentrations. Other terpenes such as sesquiterpenes and homoterpenes are emitted from cabbage damaged by herbivores (Vuorinen *et al*., 2004). The only known terpene-related compounds in *Arabidopsis* are primary metabolites or hormones such as gibberellic acids, brassinosteroids and carotenoids (Kliebenstein, 2004). Many authors have identified brassinosteroids such as 24-epicastasterone and 24-epibrassinolide in *Arabidopsis* and demonstrated their role in plants in regulation of many aspects of plant growth and development including cell division and expansion, vascular differentiation, root elongation and shoot growth (Müssig *et al*., 2003; Caño-Delgado *et al*., 2004; Bao *et al*., 2004).

2.3.3 Glucosinolates

The primary function of glucosinolates in plants is well-known as defense against different pathogenic organisms (Mithen *et al*., 1987; Milford *et al*., 1989; Blaževic and Mastelić, 2009). Other functions that have been suggested are intermediates in nitrogen and sulfur metabolism, growth regulators (Bones, 1990), plant defense against stress of extreme temperatures in cold and warm climate (Ludwick-Müller *et al*., 2000), and enhancement of growth of plant seedlings (Kelly *et al*., 1998). The flavor and odor of *Brassica* vegetables are attributed to glucosinolates and their breakdown products such as volatile isothiocyanates, thiocyanates and nitrils (Kim *et al*., 2002) (**Figure 2.1C**). Glucosinolate content in *Brassica* and *Arabidopsis* is dependent on several factors such as variety, age, the growing season, plant parts and environmental conditions (Carlson *et al*., 1987; Mithen *et al*., 1987; Rosa *et al*., 1996; Rosa *et al*., 1997; Zrybko *et al*., 1997; Kushad *et al*., 1999; Brown *et al*., 2003; Zhang *et al*., 2008; Blaževic and Mastelić, 2009).

More than 120 different glucosinolates have been identified; most of them are from the family Brassicaceae and several closely related families (Fahey *et al*., 2001; Brown *et al*., 2003). Because glucosinolates play important roles in plant-herbivore and plant-pathogen interactions (Mithen *et al*., 1987; Milford *et al*., 1989; Bennett and Wallsgrove, 1994; Doughty *et al*., 1996), many efforts have been made to identify glucosinolates in different plants species and parts (Zrybko *et al*., 1997; Reichelt *et al*., 2002; Kushad *et al*., 2004). Twenty four glucosinolates were identified from *Arabidopsis* and investigations of wild types and mutant lines of *Arabidopsis* has lead to identification of an additional 12 glucosinolates including six novel compounds. Four of these novel compounds are benzoate esters isolated from the seeds of *Arabidopsis,* the other two compounds are 3-methylbutyl- and 4-methylpentyl-glucosinolates (Reichelt *et al*., 2002) (**Table 2.1**).

The glucosinolate content of the flower buds of Portuguese *Brassica* crops (*B. oleracea* var. *tronchuda* and var. *acephala*) and *B. rapa* was studied (Rosa, 1997a). The following glucosinolates were found in the evaluated plants: glucoiberin, progoitrin, sinigrin, gluconapin, glucobrassicanapin, glucobrassicin, gluconasturtiin, neoglucobrassicin and 4-methox-glucobrassicin (**Table 2.1**). Glucobrassicanapin was exclusive for *B. rapa*, while 4-methoxy-glucobrassicin was found only in *B. oleracea*. The major glucosinolates in *B. oleracea* were sinigrin, glucobrassicin, and glucoiberin, which represent an average of 35, 29, and 25 % of the total glucosinolate content, respectively. Gluconapin represented 86 % of the total glucosinolates in *B. rapa* with glucobrassicanapin and gluconasturtiin being the other major glucosinolates. *Brassica rapa* is characterized by low amounts of indole glucosinolates (3% of the total glucosinolate content) (Rosa, 1997a). Six glucosinolates were determined and identified in wild and domestic mustard *B. hirta* and *B. juncea* using high-performance liquid chromatography coupled with electrospray mass spectrometry and photodiode-array detection. These glucosinolates were sinigrin, progoitrin, glucobarbarin, gluconasturtiin and sinalbin (Zrybko *et al*., 1997) (**Table 2.1** and **Figure 2.1B**).

Table 2.1. Distribution of glucosinolates and phytoalexins in *Arabidopsis* and *Brassica species*.

Glucosinolates	
Chemical name	**Common name**
3-Methylthiopropyl	Glucoiberverin
4-Methylthiobutyl	Glucoerucin
5-Methylthiopentyl	Glucoberteroin
6-Methylthiohexyl	Glucolesquerellin
7-Methylthioheptyl	
8-Methylthiooctyl	
2-Methylsulfinylethyl	
3-Methylsulfinylpropyl	Glucoiberin
4-Methylsulfinylbutyl	Glucoraphanin
5-Methylsulfinylpentyl	Glucoalyssin
6-Methylsulfinylhexyl	Glucohesperin
7-Methylsulfinylheptyl	Glucoibarin
8-Methylsulfinyloctyl	Glucohirsutin
4-Methylsulfinyl-3-butenyl	Glucoraphenin
3-Hydroxypropyl	
4-Hydroxybutyl	
2-Hydroxy-3-butenyl	Progoitrin
2- Propenyl	Sinigrin
3- Butenyl	Gluconapin
4-Pentenyl	Glucobrassicanapin
1-Pentenyl	
4-Hydroxy-4-pentenyl	Gluconapoleiferin
1-Methylethyl	Glucoputranjivin
1-Methylpropyl	Glucojiabutin
3-Methylbutyl	
4-Methylpentyl	
Benzyl glucosinolates	Glucotropelin
2-Phenylethyl	Gluconasturtiin
4-Hydroxybenzyl	Sinalbin
2-Benzoyloxyethyl	
3-Benzoyloxypropyl	Glucomalcomiin
4-Benzoyloxybutyl	
5-Benzoyloxy pentyl	
6-Benzoyloxy hexyl	
2-Benzoyloxy-3-butenyl	
6^{-}-Benzoyl-4-methylthiobutyl	
6^{-}-Benzoyl-4-methyl sulfinyl butyl	
6^{-}-Benzoyl-4-benzoyloxybutyl	
Indole-3-ylmethyl	Glucobrassicin
1-Methoxyindole-3-ylmethyl	Neoglucobrassicin
4-Methoxyindole-3-ylmethyl	4-Methoxy-glucobrassicin
4-Hydroxy indol-3-ylmethyl	4-Hydroxy-glucobrassicin

Phytoalexins
Brassinin
1.Methoxybrassinin
1-Methoxybrassitin
1-Methoxybrassenin B
Cyclobrassinin sulfoxide
Dehydro-4-Methoxycyclobrassinin
1-Methoxyspirobrassinin
1-Methoxyspirobrassinol methyl ether
Brassilexin
Sinalexin
Brassicanal B
Camalexin
1-Methylcamalexin
Brassitin
4-Methoxybrassinin
1-Methoxybrassenin A
Cyclobrassinin
Cyclobrassinone
Spirobrassinin
1-Methoxyspirobrassinol
Dioxibrassinin
Methyl 1-methoxyindole-3-carboxylate
Brassicanal A
Brassicanal C
6-Methoxycamalexin

Sinigrin, progoitrin, and sinalbin were found at the highest level in the flowers of the plant and the concentration decreased from flowers to the roots, the roots having the lowest concentration of glucosinolates (Zrybko *et al*., 1997). Eighteen different glucosinolates belonging to different types (aliphatic, aromatic and indolic glucosinolates) from *B. napus* var. *napus* and *B. rapa* var. *silvestris* were identified (Griffiths *et al*., 2001). Distribution of sinigrin and glucoraphanin was determined in seeds and seedlings of *Brassica* species: *B. oleracea, B. nigra, B. juncea, B. rapa*, and *B. napus*. Similar concentrations of sinigrin were detected in seedlings and seeds of the studied species but glucoraphanin concentration was lower in seedlings than in seeds of the tested species. High concentrations of sinigrin were detected in *B. nigra, B. juncea* (var. *rugosa*) and *B. oleracea* (Brussels sprouts, cabbage, cauliflower and Chinese broccoli). However, sinigrin was absent from *B. napus* and *B. rapa*. Glucosinolates contents in ornamental cabbage and kale were quantified and fifteen glucosinolates were detected varying in concentration. From these glucosinolates, progoitrin, sinigrin, glucoraphanin and glucobrassicin recorded higher concentrations

than other glucosinolates (0.5 μmolg^{-1} dry weight) (Kushad *et al*., 2004). Glucoraphanin was detected in high concentrations in *B. oleracea* var. *italica* and in moderate concentrations in *B. oleracea* var. *capitata* (cabbage) and was not detected in *B. nigra, B. rapa, B. juncea* and *B. napus* (Rangkadilok *et al*., 2002). Near-infrared spectroscopy (NIS) was used to screen and quantify the total glucosinolate (T-GSL), and also, the aliphatic glucosinolates gluconapin, glucobrassicanapin, progoitrin, glucoalyssin and the indolic glucosinolate glucobrassicin in *B. napus* ssp. *pabularia* (Font *et al*., 2005). Sulforaphane was separated and quantified in broccoli using solid phase extraction (SPE) and reverse phase HPLC. Small quantities of sulforaphane were found in the florets and stalks but the highest amounts of sulforaphane were found in the leaves (Bertelli *et al*., 1998).

The accumulation of glucosinolates can be induced after wounding and pathogen attack (Doughty *et al*., 1991; Bodnaryk, 1992), after heavy metals application (Jahangir *et al*., 2008b) as well as after treatment with salicylic acid, jasmonic acid and methyl jasmonate (Bodnaryk, 1994; Kiddle *et al*., 1994; Ludwig-Müller *et al*., 1997; Liang *et al*., 2006b). When tissues are infected, endogenous plant enzymes (thioglucosidases or myrosinases) hydrolyse the glucosinolates to unstable aglycones, which rearrange to yield a variety of products including isothiocyanates, thiocyanates and nitriles (**Figure 2.1C**). The nature of the products depends on the hydrolysis conditions and the particular glucosinolate (Fenwick *et al*., 1983; Poulton and Moller, 1993; Bending and Lincoln, 1999; Botti *et al*., 1995; Manici *et al*., 2000; Mithen, 2001; Chen and Andreasson, 2001; Al-Turki and Dick, 2003). The activity of these compounds and their products against various species of microorganisms has been documented by many investigators (Sisti *et al*., 2003). Glucosinolates are usually present in the leaves of *Brassica ssp*. at concentrations that can prevent the development of pathogens under bioassay conditions (Doughty *et al*., 1996). *Brassica rapa* contains high amounts of alkenyl glucosinolates which can be catabolized to volatile isothiocyanates or nitriles.

When *B. rapa* seedlings were inoculated with the fungal pathogen *Alternaria brassicae*, the glucosinolates were catabolized during the infection and 3-butenyl and 4-pentyl isothiocyanates were released together with dimethyl disulphide, dimethyl trisulphide, and 4-oxoisophorone (Doughty *et al*., 1996). Monitoring of the total glucosinolate content in both resistant and susceptible varieties of cabbage (*B. compestris* ssp. *pekinesis*) during the development of club root disease caused by

Plasmodiophora brassicae revealed that the glucosinolates content was significantly lower in the resistant varieties compared to the susceptible ones. The susceptible varieties showed a very high content of aliphatic glucosinolates while the resistant varieties showed high content of aromatic glucosinolates. Treatment of both varieties with salicylic or jasmonic acid showed an increase in the total amounts of glucosinolates with a different response of both varieties between the two treatments. Jasmonic acid induced indole glucosinolates production in the leaves while salicylic acid induced indole glucosinolates in both leaves and roots of both varieties (Ludwig-Müller *et al*., 1997). Studying the glucosinolate contents in leaves and cotyledons revealed that, in *B. napus* (glucobrassicin), *B. rapa* (4-hydroxy-glucobrassicin) and *B. juncea* (4-hydroxy-glucobrassicin and glucobrassicin) glucosinolates increased up to 20-fold after treatment of leaves and cotyledons with jasmonic acid (JA) or methyl jasmonate (MJ) (Bodnaryk, 1994).

Although, a quantitative and qualitative screening of glucosinolates and their byproducts has been done with different accessions of *Brassica* species (*napus, oleracea* and *juncea*), very few studies have been done on the variation for a specific cultivar of *B. rapa*. Due to the importance of *B. rapa* as a crop plant and as a wild plant with a world wide distribution, the glucosinolate content under different conditions such as healthy versus infected plants is of great interest to learn more about resistance against various pests and diseases.

2.4 Biological activity of phytochemicals

Allelopathic and autopathic potentials of plants on the growth of other plants and pathogenic fungi have been documented for many plants belonging to different families (Hegazy *et al*., 1990; Hegazy and Elsubaely, 1994; Hegazy and Fadl-Allah, 1995; Gonzalez *et al*., 1995; El-Khatib, 1997; Bagghi *et al*., 1997; El-Khatib and Hegazy, 1999; El-Khatib, 2000; Bajwa *et al*., 2002; Bajwa *et al*., 2003). However, very few investigations have been made on the allelopathic effect of Brassicaceae plants on other plants and on rhizospheric fungi (Sisti *et al*., 2003; El-Khatib and Abd-Elaah, 1998). The allelopathic potential of *Zilla spinosa* (Brassicaceae) on growth of other associated flowering plants and some rhizospheric fungi was studied. The growth inhibition of some plants and reduction in rhizospheric mycelial growth was attributed to the allelopathic potential of *Zilla spinosa* (El-Khatib and Abd-Elaah, 1998). The effect of the crude aqueous extract of *B. oleracea* var. *botrytis* leaves on

the growth and development of the pathogenic fungus, *Candida albicans* was studied. The crude aqueous juice of leaves inhibited the growth of blastocladia and reduced the appearance of *C. albicans* germ tubes. Furthermore, the extract inhibited the growth of some pathogenic filamentous fungi (Sisti *et al*., 2003).

The interaction of some members of Brassicaceae with their microbial and fungal pathogens is attributable to the wide range of biological activities of the glucosinolates and their catabolites (Fenwick *et al*., 1983; Bennett and Wallsgrove, 1994; Brown and Morra, 1997; Edwards and Brabban, 1995; Fleming and Kyung, 1997; Smolinska *et al*., 1997a,b; Olivier *et al*., 1999). Involvement of glucosinolates in resistance to pathogens was demonstrated by many researchers (Bennett and Wallsgrove, 1994; Doughty *et al*., 1996; Ludwig-Müller *et al*., 1997).

The antimicrobial activity of allyl glucosinolates on the growth of two fungi (*Helminthosporium solani* and *Veticillium dahlia*) was screened. The study indicated that these compounds inhibited the radial growth of the mentioned fungi (Olivier *et al*., 1999). Indole-3-acetonitrile from rapeseed is the major fungal inhibitor which significantly inhibits the virulent *P. lingam* and *Rhizoctonia solani*, but affects much less the growth of *Sclerotinia sclerotiorum* and does not appear to inhibit the growth of *A. brassicae* (Olivier *et al*., 1999; Pedras *et al*., 2002). Enzymatic hydrolysis of glucosinolates by myrosinase produces volatile isothiocyanates (ITCs), oxazolidinethiones, ionic thiocyanate (SCN^-) and organic cyanides. ITCs are generally regarded as the most toxic products of glucosinolate hydrolysis. The antimicrobial activities of the byproducts of glucosinolates were studied (Brown and Morra, 1997; Edwards and Brabban, 1995; Fleming and Kyung, 1997; Smolinska *et al*., 1997a,b). The glucosinolate byproducts from seed meal of *B. napus* inhibited the growth of *Aphanomyces* (Smolinska *et al*., 1997a,b). The antimicrobial activity of 2-phenylethyl isothiocyanates was studied. The results documented the effect of the isothiocyanates at low concentration on the diversity of the microbial community in the rhizosphere of canola (Rumberger and Marschner, 2003).

Different fractions of *Arabidopsis thaliana* leaves were studied for their antimicrobial activity. 4-Methylsulfinylbutyl isothiocyanate was purified and its antimicrobial activity has been determined. This isothiocyanate was found to inhibit *Pseudomonas syringae* growth at a concentration of 28 μM (Tierens *et al*., 2001). Toxicity of camalexin against fungi and bacteria was reported by several research groups (Pedras *et al*., 1998; Mert-Turk *et al*., 2003).

In an attempt to reveal the role of the phytoalexin camalexin in the disease resistance of economically valuable *Brassica* crops, e.g. canola/rapeseed (*B. napus, B. rapa*) and mustard (*B. juncea*), the antimicrobial activity of camalexin against *Phoma lingam, A. brassicae, P. syringae, P. cichorii, Erwinia carotovora* and *Xanthomonas campestris* was studied. Camalexin inhibited both fungal and bacterial growth (Pedras *et al*., 1998).

Allelopathic activity of some flavonoids in different plant species was studied by several research groups. The allelopathic potential of quercetin and seven derivatives on *Arabidopsis* seedling growth and *Neurospora crassa* conidia germination was screened (Parvez *et al*., 2004). Quercetin and its derivatives had the potential to inhibit the growth of *Arabidopsis* seedlings but only quercetin 3-methyl ester and its glucosides had the potential to inhibit the germination of conidia in *N. crassa*. The effect of quercetin 3-methyl ester and its glucosides on inhibition of the germination of conidia in *N. crassa* was attributable to the presence of a methyl group in the flavonoid nucleus (Parvez *et al*., 2004). The antibacterial activity of flavonoids isolated from *Combretum erythrophyllum* (Combretaceae) was tested. Apigenin, genkwanin, 5-hydroxy-7,4´-dimethoxyflavone, kaempferol, rhamnocitrin, and quercetin-5,3´-dimethylether had good antibacterial activity against *Vibrio cholerae*, and *Entercoccus faecalis* with minimum inhibition concentration (MIC) values ranging from 25-50 µg/ml and against *Pseudomonas aeruginosa, Escherichia coli* with MIC values ranging from 50-100 µg/ml. From these flavonoids, rhamnocitrin and quercetin-5,3´-dimethylether inhibited the growth of *Micrococcus luteus* and *Shigella sonei* at concentration of 25 µg/ml (Martini *et al*., 2004). *Aspergillus niger* was resistant to all last mentioned compounds at 100 µg/ml, the highest concentration tested (Martini *et al*., 2004). The effect of oxylipines on the growth and development of some pathogenic fungi associated with *B. napus* was reported.

The oxylipines inhibited the germination of *L. maculans* spores (Granér *et al*., 2003). This study suggested that selected oxylipines may be used for disease control in *Brassica* plants.

2.5 *Brassica* defense

Plants are fixed organisms growing in substrates, but like all other living organisms they have adaptive mechanisms by which they can respond to different environmental stresses such as nutrient deficiencies, toxic xenobiotics, and pathogen

infections (Matsumoto *et al*., 2004). Pathogen attack affects not only plant development but also may have a strong negative impact on the quality and quantity of crops (Pedras *et al*., 2002). Plants have an enormous and complex arsenal of defense mechanisms to fight pathogen attack. Biosynthesis of secondary metabolites is the most significant aspect of these defense mechanisms (Pedras *et al*., 2002) **(Figure 2.2)**.

The host types of interaction are divided into compatible and incompatible interactions. In compatible interactions the susceptible plant becomes diseased upon attack by a virulent pathogen, while in incompatible interaction the resistant plant does not develop disease upon attack by an avirulent pathogen (Laugé and De Wit, 1998). In addition to mechanical and chemical barriers set up by the plant during infection with fungal spores, another new weapon appears to stop the fungal penetration and development. This weapon is known as systemic acquired resistance (SAR) (Ryals *et al*., 1994) by which the attacked plant can systematically biosynthesize antifungal secondary metabolites which are not produced by healthy plants.

Some compounds are very important for inducing defense against pathogens. The role of ethylene, salicylic acid (SA), the SA-mimicking compound 2,4-dichloroisonicotonic acid (INA), and jasmonates in plant responses to pathogens are well documented (Gaffney *et al*., 1993; Delaney *et al*., 1994; Chen *et al*., 1995; Dong, 1998; Heil and Bostock, 2002; Zhang and Shapiro, 2002; Shah, 2003; Durrant and Dong, 2004). Systemic acquired resistance (SAR) is characterized in plants such as *Arabidopsis* by the marked reduction in susceptibility to disease resulting from prior infection by an avirulent pathogen. Pre-inoculation of plants with a pathogen that induces necrosis leads to the development of systemic acquired resistance (SAR) to subsequent pathogen attack (Ward *et al*., 1991; Ryals *et al*., 1994; Delaney *et al*., 1995). Induction of SAR is dependent largely on the hormone salicylic acid (SA), which is essential for the activation of local resistance and SAR genes and gene-for-gene resistance, and for limiting disease caused by virulent pathogens (Gaffney *et al*., 1993; Delaney *et al*., 1994, 1995; Delaney, 1997; Mauch-Mani and Métraux, 1998).

External application of either (SA) or (INA) to wild type *Arabidopsis* leads to the expression of genes of some proteins and increased resistance to *Peronospora parasitica* and *P. syringae* (Uknes *et al*., 1992; Cao *et al*., 1994; Delaney *et al*., 1994). Involvement of SA in plant programmed cell death (PCD) was demonstrated in

lesions simulating disease mutants (*lsd*) by introduction of bacterial salicylate hydroxylase gene, which suppressed the necrotic lesion phenotype (Weymann *et al*., 1995). The UDP glucosyl transferase (UGT 73B5) gene is induced in *Arabidopsis* leaves during PCD stimulated by super oxide (O^{-}_{2}) in the presence of cycloheximide (CHX) or salicylic acid (SA). UGT73B5 transcription was also induced by mechanical cell crushing and by infection with an avirulent strain of *P. syringae* or by treatment with intercellular fluid from *Arabidopsis* cell culture infected with *Botrytis cinerea* (Mazel and Levine, 2002).

JA is a crucial component of the resistance response of *Arabidopsis* roots to *Pythium* root rot (Vijayan *et al*., 1998) and in rice wounding results in elevated JA levels and systemic resistance to microbial infection (Schweizer *et al*., 1998). Treatment of *Arabidopsis* with methyl jasmonate reduces the disease development caused by several fungi such as *Alternaria brassicicola, B. cinerea* and *Plectosphaerella cucumerina* (Thomma *et al*., 2000). The relation between JA, SA and SAR was documented by many researchers. Tobacco plants with high-SA/low-JA concentrations show higher SAR and lower levels of induced insect resistance (IR). Conversely, low-SA/high-JA plants have decreased SAR and enhanced insect resistance (Felton *et al*., 1999).

Phytoalexins are part of the inducible plant defense against microorganisms. Plant resistance against pathogens thus, among others, depends on the level of phytoalexins produced (Subba Rao and Strang, 1994). The phytoalexins accumulated in infected sites are considered an important factor in plant disease resistance (Pedras *et al*., 1998; Kuc, 1995; Smith, 1996; Heath, 2000). The role of phytoalexins in stopping pathogen development in host tissue is far from clear for many host pathogen interactions. Although a microbial infection may result in the production of phytoalexins, these compounds not always have measurable antimicrobial effects against pathogenic fungi. Also the mechanism of detoxification of some compounds such as camalexin by some fungi such as *S. sclerotiorum* and *R. solani* (Pedras and Khan, 1997) is not clear and needs more study. Synergism of various compounds and enzymes may play an important role in resistance.

The indolic phytoalexin, camalexin is induced by pathogen attack and by elicitation with abiotic factors that also generate the reactive oxygen species (ROS) in plants. It is considered the principal and may be the only phytoalexin in *Arabidopsis* (Kliebenstein, 2004; Tsuji *et al*., 1992; Zook, 1998; Morrissey and Osbourn, 1999).

The accumulation pattern of camalexin, in several ecotypes of *Arabidopsis* after inoculation with virulent and avirulent strains of *Hyaloperonospora parasitica* or exposure to UV irradiation was studied. The ecotype Ws accumulated largest camalexin after inoculation and the ecotype Columbia accumulated the smallest amount. The mutation has little effect on the resistance or susceptibility to this pathogen and the camalexin accumulation is not a major determinant for defense against *H. parasitica* (Mert-Turk *et al*., 2003). A phytoalexins-deficient mutant of *A. thaliana* showed significantly higher susceptibility to the infection by the fungus *A. brassicicola* than the wild-type plants (Hain *et al*., 1993; Thomma *et al*., 1999; Mert-Turk *et al*., 2003). Using the biosynthetic mutant of *Arabidopsis* (*pad-3*) provided evidence that camalexin accumulation is important in resistance of *Arabidopsis* to *A. brassicicola* but not to *B. cinerea* (Thomma *et al*., 1999). In resistance, one must also consider the possibility, that a pathogen is able to overcome some of the defense mechanisms of the plant, e.g. by detoxification of phytoalexins (Pedras and Ahiahonu, 2005). Phytoalexins synthesis is most likely a general response to infection and resistance is also attributed to the presence of some proteins that regulate the defense response (Sisti *et al*., 2003).

The leaves of *Arabis lyrata* were inoculated with either the bacterial pathogen *Pseudomonas syringae pv. maculicola* strain ES4326 or *Cochliobolous carbonum*, a fungal nonpathogen for *A. lyrata.* The results obtained using high resolution mass spectroscopy and ^{1}H-NMR indicated the accumulation of camalexin, a phytoalexin known from *Arabidopsis* (Zook *et al*., 1998). Three phytoalexins (brassinin, methoxybrassinin and cyclobrassinin) were isolated from Chinese cabbage (*B. compestris* ssp. *pekinensis*) inoculated with the bacterium *Pseudomonas cichorii*. These compounds have structurally unique features that include an indole ring with sulphur atoms (Monde *et al*., 1995) (**Figure 2.1A**). Although the search for phytoalexins as antifungal compounds from Brassicaceae members has started long times ago (Tsuji *et al*., 1992; Zook *et al*., 1998), the phytoalexins of some species have not been studied yet. The importance of *B. napus, B. oleracea* and *B. juncea* as a source of oil and as valuable vegetables enriched with protein for human and animals, has pushed the researchers to study the interaction between pathogens and these species searching for antimicrobial phytoalexins. However, very few studies have been done on wild plants of this family. *Brassica rapa*-fungus interaction has not been studied yet in spite of the importance of *B. rapa*.

2.6 Fungi

Chemistry of phytotoxins

During the coevolution between host plant and pathogens, fungi have developed their own biochemical strategies to survive. Some pathogens have developed different ways to detoxify phytoalexins by means of some enzymes which convert these compounds to less toxic derivatives, others have developed mechanisms to suppress the defense response of the host plant making the plant unable to prevent the entering of the pathogen (Pedras *et al*., 2002). Certain fungal pathogens, especially in the genus *Alternaria*, produce low molecular weight compounds known as host-specific or host selective toxins (HSTs) that determine their host range and contribute to their virulence or pathogenicity (Pedras *et al*., 2000). HSTs are very useful for the study of the mechanism underlying host specificity in parasitism, being reliable substitutes for the pathogens that produce them (Otani *et al*., 1995).

Nine examples of HST-producing pathogens have been documented and many *Alternaria* HSTs have been isolated and identified (Otani *et al*., 1998; Mackinnon *et al*., 1999). Inoculation of *Arabidopsis* leaves with conidial suspension of the necrotrophic fungus *B. cinerea* results in the production of a new phytotoxin (botrydial) (Deighton *et al*., 2001) (**Figure 2.3**). The highly virulent isolates from the blackleg fungus *P. lingam* produce the phytotoxins phomalide and sirodesmins (**Figure 2.3**), while avirulent isolates produce metabolites with low phytotoxicity such as phomapyrone A (Pedras *et al*., 1999; Pedras and Chumala, 2005). Phomalairdenone, a new host-selective phytotoxin was isolated and elucidated from the virulent isolates of the blackleg fungus, *P. lingam* (Pedras *et al*., 1999). Recently phomapyrones D-G, phomenin B, infectopyrone and polanarizine B and C were isolated from the fungal pathogen *L. maculans*. The biosynthesis pathways and the biological activity of these phomapyrones were discussed (Pedras and Chumala, 2005).

The long term study of the interaction between the fungus *L. maculans* and *Brassica* plants revealed that the sirodesmin, phomalirazine, and phomalide are the major and the most important phytotoxic compounds from *L. maculans* (Pedras *et al*., 1990; Pedras and Biesenthal, 2000).

The fungus *A. brassicae* causing black leaf spots to brassicas, produces in culture an HST which is toxic only to *Brassica spp*. Its structure is identical to a cyclic peptide, destruxin B, which is also produced by *Metarhiziun anisopiae*, a fungus pathogenic on insects. Brassicaceae plants transform the host-selective toxin destruxin B into hydroxydestruxin and its β-D-glucosyl derivative (Pedras *et al*., 2003).

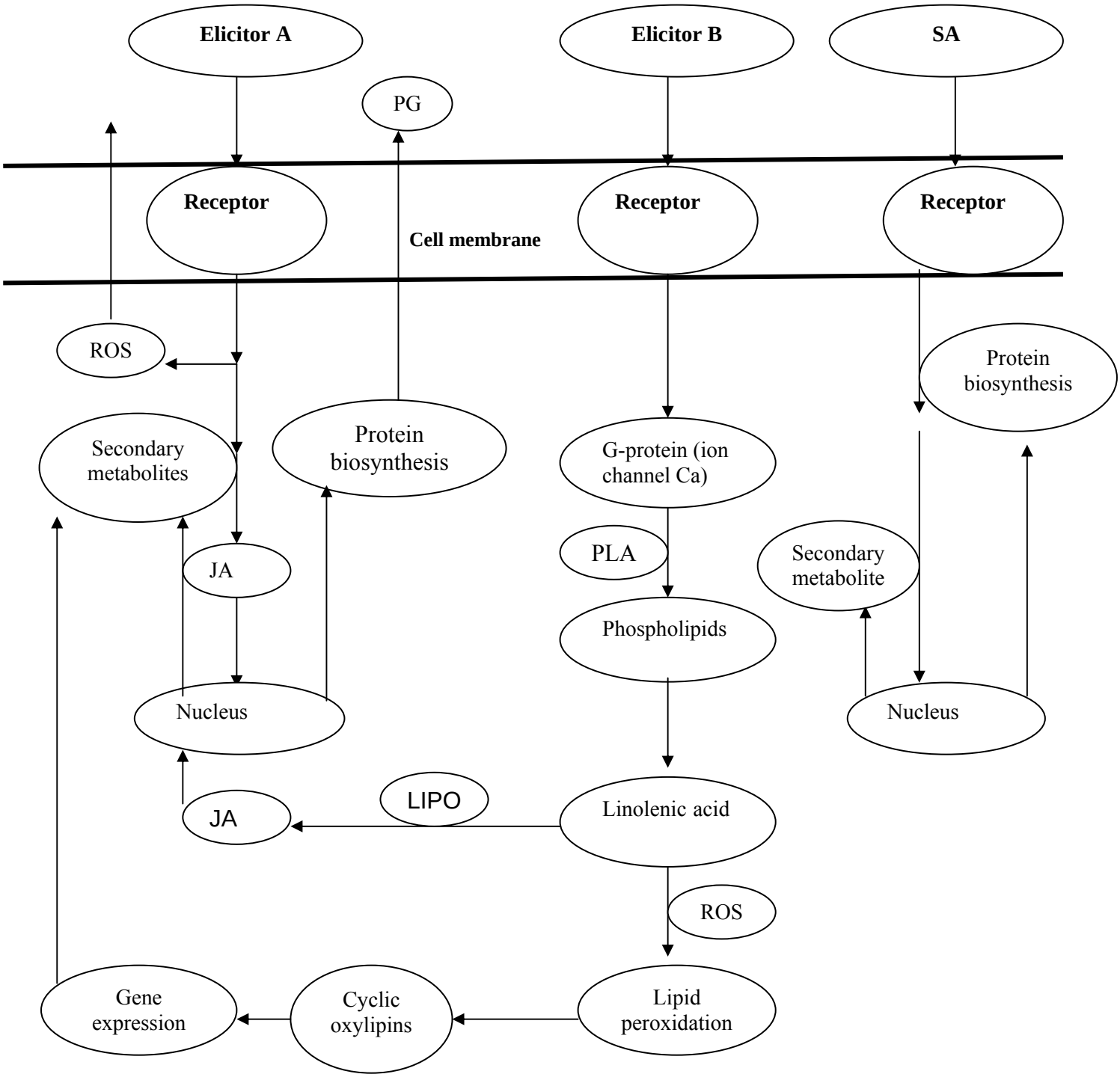

Figure 2.2. Scheme for biosynthesis of defensive secondary metabolites in plants, LIPO: lipoxygenase, PG: polygalacturonase, PLA: phospholipase A, ROS: reactive oxygen species, SA: salicylic acid, JA: jasmonic acid.

The phytotoxicity of destruxin B was demonstrated together with four other natural phytotoxic analogs using leaf uptake and leaf puncture/scratch assays on resistant and susceptible plant species (Pedras *et al*., 2000). Homodestruxin B was the most toxic of the five compounds, followed by destruxin B and desmethyldestruxin B. Hydroxydestruxin B was less toxic than the parent toxin (Pedras *et al*., 2000) (**Figure 2.3**). The phytotoxicity of destruxin B was found to vary from species to species, for example, *B. juncea* cv. Lethbridge was more sensitive to destruxin B than *B. napus* cv. Altex (Bains and Tewari, 1987; Buchwaldt and Green, 1992).

Methanol-soluble extract from the fungus *A. brassicicola* contains toxic substances to *Brassica spp*. but these toxins were not specific in their activity toward plant species (MacDonard and Ingram, 1986). *Alternaria brassicicola* produces host-specific toxins and releases them during spore germination on the leaves of *Brassica spp.* Theses toxins have been tested and the protein nature of these toxins have been demonstrated. These are the first toxins described among many host-specific toxins produced by *Alternaria species* (Otani *et al*., 1998). Six new fusicoccane-like diterpenoids (brassicicene) have been isolated from the liquid culture filtrate of the canola pathogen *A. brassicicola* (Mackinnon *et al*., 1999) (**Figure 2.3**). Nine novel tricycloalternarene compounds were isolated and elucidated from the culture filtrate of *A. alternate* isolated from *B. sinensis* (Nussbaum *et al*., 1999).

The metabolism of diverse cruciferous phytoalexins by agriculturally important pathogens was investigated (**Figure 2.4**). The virulent strains of the blackleg fungus can rapidly detoxify brassinin, brassicanal, brassilexin and cyclobrassinin while camalexin did not appear to be metabolized by *P. lingam* (Pedras and Khan, 1997). *Rhizoctonia solani* has the ability to metabolize camalexin into metabolites that were not inhibitory against *R. solani*, indicating that camalexin was detoxified (Pedras and Khan, 1997; Pedras and Ahiahonu, 2005). *Rhizoctonia solani* has the ability to metabolize and detoxify camalexin while *A. brassicae* or *P. lingam* can not, which allows the accumulation of camalexin in plants infected with *A. brassicae* and *P. lingam*, explaining the higher resistance of members of Brassicaceae to *A. brassicae* and *P. lingam* than to *R. solani* (Pedras and Khan, 1996; Pedras and Okanga, 1998, 1999; Pedras *et al*., 1998; Pedras and Ahiahonu, 2005).

The investigation of phytoalexin detoxification in fungal pathogens is of great importance, not only because it allows a better understanding of the interaction between plant and pathogen, but also because this process can be exploited to deter

pathogenecity. The detoxification of phytoalexins by fungal pathogens could be utilized to control pathogenic fungi, if inhibitors of those fungal detoxifying enzymes were available (Pedras *et al*., 1997b).

Not only does the pathogen transform the secondary metabolites and especially phytoalexins into nontoxic compounds, but also the plant can metabolize the phytotoxic compounds of pathogenic fungi and transform them to less toxic compounds. The blackspot-resistant species (*Sinapis alba*) metabolizes the major phytotoxin produced by *A. brassicae* (destruxin B) to less toxic products, hydroxydestruxin B and β-D-glucosyl hydroxyl destruxin B. Hydroxydestruxin B induces the biosynthesis of phytoalexins in blackspot-resistant species. This is a very important example of phytotoxic detoxification and simultaneous phytoalexin elicitation by the detoxification (Pedras *et al*., 2001). Some toxin-producing fungi are able to degrade or transform their own products under suitable conditions such as using these toxins as a source of energy under starvation (Karlovsky, 1999). *Aspergillus flavus* and *A. parasiticus* can degrade aflatoxins and fusaric acid was detoxified by its producers *Fusarium oxysporum* and *Gibberella fujikuroi* into 5-*N*-(3-hydroxybutyl)-pyridine-2-carboxylic acid with a significantly reduced phytotoxicity (Karlovsky, 1999).

It will be desirable if we could exploit the detoxification trait in plant breeding programs for engineering disease resistance in crop plants. Thus, understanding of the fungal phytotoxin synthesis and transformation of phytoalexins and other secondary metabolites by the fungi and *vice-versa*, the detoxification of fungal secondary metabolites and host-selective toxins will be important to develop novel approaches for crop protection and plant breeding including genetic engineering.

2.7 Plant-fungal interaction

Although plants are fixed organisms, they have their own defense weapons and they are resistant against most fungi except for a few pathogens that have developed resistance. The interaction between plants and their pathogens is complicated and may be very specific. On the one hand, the defense mechanism of plants against their pathogens includes the use of antifungal chemicals such as phytoalexins, and phytoanticipins, e.g. inducing glucosinolates and flavonoids.

On the other hand, pathogens have developed some mechanisms to detoxify these chemicals and their antimicrobial activities which enable them to penetrate the plant tissue and colonize the plant (Grayer and Kokubun, 2001). The methods for studying the plant-pathogen interaction have changed from traditional techniques which depend on visual scoring of symptoms (scoring either lesion diameter or conidia formation) into modern techniques which rely on using sophisticated equipment by which we can monitor the disease stages and fungal development and easily quantify the growth of the pathogen in infected plants, such as real time quantitative PCR and proteomics (Zimmerli *et al*., 2004; Gachon and Saindrenan, 2004). Recently real-time PCR was used to monitor quantitatively the growth of the fungus *A. brassicicola* and *B. cinerea* in *Arabidopsis* leaves by quantification of fungal and plant DNA in infected leaves (Gachon and Saindrenan, 2004).

Many research groups have studied *Arabidopsis* as a model system for host parasite interaction, e.g. enhancement of resistance to powdery mildew pathogens and genetic analysis of disease resistance (Xiao *et al*., 1997; Frye and Innes, 1998; Vogel and Somerville, 2002). Silicon was applied to *Arabidopsis* inoculated with the powdery mildew fungus, *Erysiphe cichoracearum* to examine if silicon application will reduce the level of disease in *A. thaliana* and to evaluate the ultrastructure of *E. cichoracearum-Arabidopsis* interaction on the leaves of plants pretreated with silicon. The results have established the *Arabidopsis-Erysiphe cichoracearum* pathosystem as a valid model to investigate the role of silicon in plant-microbe interactions (Ghanmi *et al*., 2004). Elemental sulfur is the only known, inorganic phytoalexin. High constitutive levels of sulfur occurred in control and hypersensitively responding leaves of *B. oleracea,* irrespective of challenge by an incompatible race of *Peronospora parasitica* and in inoculated leaves of *Arabidopsis* (Williams and Cooper, 2003). Production of sulfur in sufficient amounts, at the right time and place of some interactions refers to its contribution in resistance of some plants against some pathogenic microorganisms (Williams and Cooper, 2003).

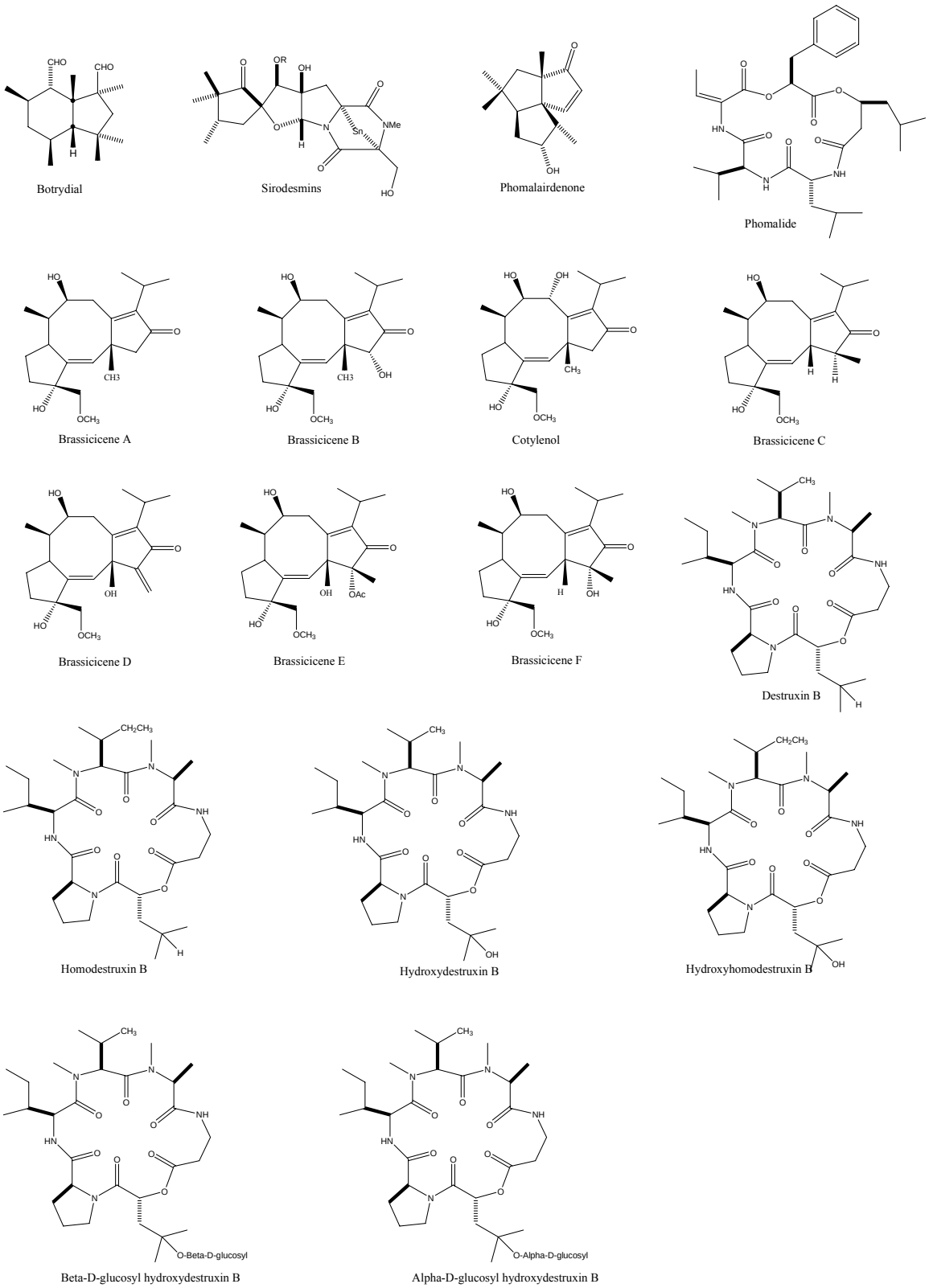

Figure 2.3. Chemical structures of some host-selective toxins and secondary metabolites of some crucifer infected fungi.

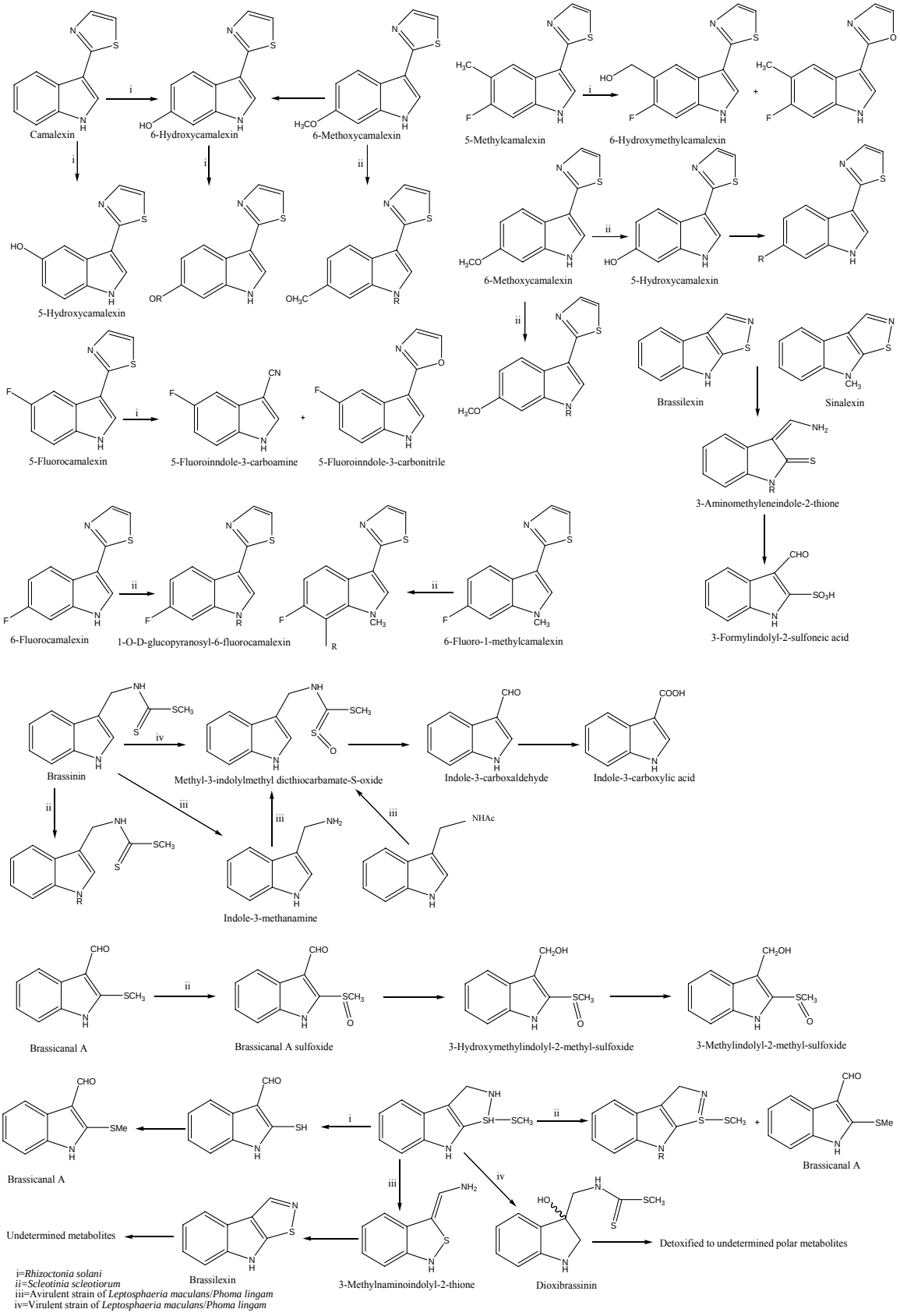

Figure 2.4. Detoxification of phytoalexins by different pathogenic fungi (Pedras and Khan, 1996; Pedras and Okanga, 1998, 1999; Pedras and Ahiahonu, 2005).

Many attempts have been made to explain the interaction between plants and pathogens. A reporter molecule would be useful for observing disease progress in general and be more accurate than the time consuming staining and fixing techniques that do not readily allow observation of live infected tissues. Green fluorescent protein (GFP) as a reporter has been used in a few plant-pathogen interactions including maize pathogens *Cochliobolus heterostrophus* (Maor *et al*., 1998), *Ustilago maydis* (Spelling *et al*., 1996), powdery mildew of barley (Nielsen *et al*., 1999), and the rice blast fungus *Magnaporthe grisea* (Liu and Kolattukudy, 1999). Recently the GFP was used in *Brassica-Leptosphaeria maculans* and *Arabidopsis* and barley-*Fusarium graminearum* interactions (Sexton and Howlett, 2001; Skadsen and Hohn, 2004), respectively. Strong fluorescence in combination with high protein levels in cotyledon and stem lesions of *B. napus* and *B. junceae* was reported (Sexton and Howlett, 2001). The utility of GFP in monitoring the growth pattern of the pathogen and for resistance studies utilizing the *Arabidopsis* model system was documented (Skadsen and Hohn, 2004). The interaction between *A. thaliana* and *Albugo candida* was studied and the ultrastructure for the interaction using electron microscopy was characterized. The results suggested that programmed cell death (PCD) or apoptosis is the cause or closely associated with the cause of restricted pathogen development in the highly resistant interactions (Soylu *et al*., 2003). The interaction between *L. maculans* and *B. napus* was studied (Roussel *et al*., 1999). *Brassica napus* was treated with an avirulent, and a virulent isolate of *L. maculans* and the elicitor cryptogein to monitor the hypersensitive reaction (HR) through observations of ultrastructural features of xylem and phloem parenchyma (morphological changes in nuclei, coagulation of the cytoplasm, reminiscent of cell death). The results of this study revealed that the infiltration of *B. napus* with the elicitor cryptogein or treating with avirulent isolates of *L. maculans* induces the response of vascular parenchyma cells associated with HR induction. Treatment of the plant with the virulent isolates of *L. maculans* did not reveal any of these reactions (Roussel *et al*., 1999). Monitoring the SAR in oilseed rape infected by highly virulent, weakly virulent or combination of both isolates of *L. maculans* at different intervals revealed that weakly virulent isolates can induce SAR to highly virulent isolates in oilseed rape (Mahuku *et al*., 1996). Many investigators have studied the interaction between fungi and *Brassica species* and *Arapidopsis* on the level of the proteins, genes and gene expression (Epple *et al*., 1997; Nashaat *et al*., 1997; Thomma and Broekaret, 1998; Sexton and

Howlett, 2001; Cramer and Lawrence, 2003, 2004; Li *et al*., 2005). The interaction between *Sclerotinai sclerotiorum* and a resistant *Brassica napus* cultivar was studied (Li *et al*., 2005). Expressed sequence tag analysis (EST) was used and the genes associated with fungal pathogenesis were identified. These genes encoded potential pathogenicity factors including four endopolygalacturonases, two expolygalacturonases and several metabolite transporters. Random expressed sequence tags in *Arabidopsis* have revealed several plant defense genes in this plant species (Epple *et al*., 1997). Cloning and characterization of three genes encoding host-cell-wall degrading enzymes in *L. maculans* infected *B. napus* in the search for solutions of the problem of infection of crop plants by fungi have been achieved (Sexton *et al*., 2000). EST technique was used to study the genes responsible for plant defense against pathogenic fungi in *Arabidopsis* and these genes were classified into two subfamilies according to their expression in the seedlings and their induction by methyl jasmonate, salicylate, ethephon, silver nitrate or different phytopathogenic fungi (Epple *et al*., 1997). Restriction enzyme mediated insertional mutagenesis (REMI) was used to isolate the pathogenicity genes of *L. maculans* and to study the gene silencing process in this fungus. Twelve transformants were identified with reduced pathogenicity of *B. juncea* and *B. napus* and the gene silencing process (isocitrate lyase) was understood in this fungus (Indurm and Howlett, 2003). Using suppression subtractive hybridization (SSH) between RNA isolated from *A. brassicicola* spores incubated in water and on the leaf surface of the *Arabidopsis* ecotype Landsberg, researchers identified fungal genes expressed during the infection of *Arabidopsis* by *A. brassicicola.* RT-PCR was used to examine the expression of these genes during infection of *B. oleracea* var. *capitata* (cabbage), *in vitro* growth in nutrient rich media, and infection of *Arabidopsis.* The results of this important study provided the first insight into genes expressed during *A. brassicicola* infection of *Brassica* species that may be involved in fungal pathogenesis (Cramer and Lawrence, 2004).

2.8 Conclusion

The importance of *Brassica* as a source of oil and protein of plant origin for human and animal nutrition and the complete genome sequence of *Arabidopsis* encourages researchers to study the interaction between Brassicaceae and pathogenic fungi. Understanding the plant's defense and the way some microorganisms can break through this will help to develop novel approaches to crop protection. This might be achieved through conventional breeding or genetic engineering. The present research is focused on the search for plant defense secondary metabolites instead of application of pesticides and fungicides that harm the environment. Quite a few secondary metabolites have been identified in Brassicaceae. Particularly the group of glucosinolates has attained much attention as they seem to be involved as phytoanticipins in the chemical defense of the plants. In spite of numerous investigations done on the role of phytoalexins in plant defense mechanisms, their role has not yet been elucidated completely. Camalexin was identified as a phytoalexin in several species where it has a role in plant defense. But for many other compounds the function is not known yet. The effect of some of these compounds on fungi has been studied and detoxification has been found in some cases but also in this field much is still unclear. Better understanding of this process will be important in connection with understanding the mechanism of resistance. Furthermore, the possibility of synergy between the various plant compounds and enzymes needs much more attention.

2.9 Future prospects

With the presently available genome sequence of *Arabidopsis* and the tools of molecular biology, biochemistry and phytochemistry, it will be possible to approach the above mentioned problems in a novel holistic way. By integration of the mentioned expertise via the different ´omics´ technology platforms, it shall be possible to better understand the total network of plant responses to various external factors including the regulation via various signaling pathways. Thus, for studying the various interactions of the plant with different organisms, a systems biology like approach should be developed to learn more about the possibility of synergy of different compounds and proteins in defense and about detoxification processes in the plant and the attacking organisms.

Metabolomics is an important tool for such studies. To have an unbiased view of all metabolites, HPLC, GC-MS, LC-MS and NMR spectroscopy based metabolomics to detect the compound or compounds responsible for the inhibition of the growth of fungi or inhibiting insect foraging seems very useful. Metabolite measurement combined with principal component analysis together with other advanced techniques such as proteomics and DNA microarrays will be the approach for identifying and characterizing signaling and biosynthetic pathways in defense responses. The results of such a systems biology approach will lead to the identification of genes involved in the defense of the plant or new targets in microorganisms or insects for developing crop protectants. Proteomics may also be useful in identification of proteins that are directly involved in defense, e.g. myrosinases or peroxidases. Eventually the integration of all these results should lead to recommendations for breeding programs and possibly to targets for metabolic engineering.

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