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The regulatory mechanism of fungal elicitor-induced secondary metabolite biosynthesis in medical plants

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Abstract: A wide range of external stress stimuli trigger plant cells to undergo complex network of reactions that ultimately lead to the synthesis and accumulation of secondary metabolites. Accumulation of such metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules. Throughout evolution, endophytic fungi, an important constituent in the environment of medicinal plants have known to form long-term stable and mutually beneficial symbiosis with medicinal plants. The endophytic fungal elicitor can rapidly and specifically induce the expression of specific genes in medicinal plants which can result in the activation of a series of specific secondary metabolic pathways resulting in the significant accumulation of active ingredients. Here we summarize the progress made on the mechanisms of fungal elicitor including elicitor signal recognition, signal transduction, gene expression and activation of the key enzymes and its application to this process. This paper provides guidance on studies which may be conducted to promote the efficient synthesis and accumulation of active ingredients by the endogenous fungal elicitor in medicinal plant cells, and provides new ideas and methods of studying the regulation of secondary metabolism in medicinal plants.

Keywords: endophytic fungi, elicitor, signal transduction, gene expression, secondary metabolism

Introduction:

In recent years the production of secondary metabolites with distinct and complex structures by plant cell cultures has been one of the most extensively explored areas owing to the enormous commercial value of these compounds, the limited availability or endangered status of parent plant species, and the extremely low levels of the secondary metabolites in plants (Cetin *et al.* 2014, Karuppusamy 2009, Thakore *et al.* 2015). The plant-microbe interactions and plant defense responses, as well as the signal transduction pathways involved, have been studied extensively and continue to be topics of active research and discussions (Goto *et al.* 2012, Li *et al.* 2007, Shigeri *et al.* 1992, Zeng *et al.* 2014). The use of fungal elicitors has been reported to be one of the most effective strategies for improving the productivity of useful secondary metabolites in plant cell culture (Takeuchi *et al.* 2013, Wang *et al.* 2012) and it is also one of the most effective means for the enhancement of hairy root (Chandra *et al.* 2011, Zhang *et al.* 2013). The recent studies of fungal elicitors focus on fungal elicitor recognition, G-protein, Ca²⁺ and H₂O₂ signal transduction, signal amplification of jasmonic acid (JA), nitric oxide (NO), salicylic acid (SA), abscisic acid (ABA), ethylene (ETH), signal crosstalk, gene expression, activation of the key enzymes and the application of fungal elicitor (Chen *et al.* 2015, Dewanjee *et al.* 2014, Fang *et al.* 2009, Gao *et al.* 2012). Plants produce secondary metabolites in nature as a defense mechanism against attack by pathogens and that's the reason why fungal elicitors can trigger the formation of secondary metabolites (Jeong *et al.* 2005). It is well known that phytoalexin is one of the most widely researched secondary metabolites (Pedras *et al.* 2009, Pedras *et al.* 2008). MePIP-1, the analogue of peptide elicitor PIP-1, showed a requirement of continuous elicitor stimulation for 3-6 h for the

phytoalexin production, which is likely to be regulated by long-lasting activation of MAPK (mitogen-activated protein kinase)(Kim *et al.* 2014). Fungal cell walls and fragments of elicitors can induce the formation of low and high molecular weight defense compounds in plant cell suspension cultures, suggesting that this induced synthesis requires a signal molecule transmitting the message between the elicitor plant cell wall receptor and gene activation(Mueller *et al.* 1993). Thus, the signal transduction is complicated and the application of fungal elicitors is extensively present in medical plants.

Although a number of studies have focused on the elicitation to increase several metabolites, the mechanism of fungal elicitors at both physical and molecular level has not yet been elucidated. This review highlights the mechanism of fungal elicitor-induced secondary metabolites accumulation at four levels, including signal transduction, the integration of transcription factors, gene expression and the activation of the key enzymes, and their crosstalk.

1. Fungal elicitor

Fungal elicitor is a mechanism which induces plant phytoalexin and causes plant hypersensitivity or self – defense reaction during the physiological processes of plant disease resistance (Sangeetha *et al.* 2015, Takikawa *et al.* 2015, Zhang *et al.* 2015). There is a long history of research of fungal elicitor-induced metabolite accumulation of taxol in *Taxus chinensis* (Pilger) Rehd. by activated spores of *Cytophora abiotis* and *Penicillium minioluteum* dating back to the early 1990s (reference). Different kind of fungal elicitors are applied for the metabolite synthesis in medical plants (Table 1). Different fungal elicitors may lead to various results in the same plant, for example, the biosynthesis of lignans was differentially affected by fungal elicitors and *Fusarium graminearum* extract and it induced the highest increase of podophyllotoxin (PTOX) , 190 $\mu\text{g}(-1)$ dry weight (DW), and lariciresinol, 260 $\mu\text{g} \cdot \text{g}^{-1}$ DW in *Linum album*, which was two-fold and three-fold greater than the untreated control, respectively(Bahabadi *et al.* 2014). Differential displacing activity of the glucans on *P. megasperma* elicitor binding corresponds closely to their respective ability to elicit phytoalexin production in a cotyledon bioassay(Schmidt *et al.* 1987). A growing body of evidence suggests that fungal elicitor induced secondary metabolites accumulation in plant is associated with the defense responses(Kishi - Kaboshi *et al.* 2010, Wang *et al.* 2006, Wang *et al.* 2007). In recent years, several fungal elicitors have been frequently used to induce the accumulation of secondary metabolites. The species and usage counts of different fungal elicitors used for inducing secondary metabolite accumulation in medical plants is shown in Figure1. The abscissa represents different fungal species while the ordinate represents the usage counts of fungal extracted as elicitor. The figure displays that *Fusarium*, *Pythium*, yeast, *Aspergillus*, *Penicillium* and *Trichoderma* species are frequently extracted as fungal elicitors and are used to induce secondary metabolites in medical plants. Among these fungal elicitors, *Fusarium* species are the most frequently. Although the statistical data on this species is incomplete; it does indicate that *Fusarium*, yeast, and *Pythium* species can be extracted as general fungal elicitors and may have universal and general usage in the induction of secondary metabolites in medical plants to a certain degree. As stated in Table 1, any one fungal elicitor can have different effects on different medical plants. Thus, although *Fusarium*, yeast, and *Pythium* species can be used to elicit specific secondary metabolites in various medical plants, they may not be the best elicitor for the induction of a specific secondary metabolite.

In general, fungal elicitors include the degradation products and, metabolites(Algar *et al.* 2012), secreted substances or fermented liquid of fungi. The chemical nature of fungal elicitor can

be classified into oligosaccharide, proteins and polyunsaturated fatty acids, which have been intensively researched and are discussed below:

1.1 Oligosaccharide

Oligosaccharide elicitors are derived from the β -glucans of pathogenic *Phytophthora sojae*, this has been very well characterized and a double-branched hepta- β -glucoside generated from *P. Sojae* glucan has been obtained which has been shown to be a very active elicitor for glyceollin biosynthesis in soybean cotyledon cells (Sharp *et al.* 1984). Furthermore, a pentasaccharide purified from an enzymatic digest of the β -glucan from the rice blast fungus, *Magnaporthe grisea* (*Pyricularia oryzae*) in the reduced form, shows potent elicitor activity and induces phytoalexin biosynthesis in suspension-cultured rice cells (Yamaguchi *et al.* 2000). A beta-1,3- or 1,6-oligoglucan (AaGlucan) from the fungus *Alternaria alternate* 102 shows strong elicitor activity in tobacco BY-2 cells. It has also been reported that NtMYBGR1 (a novel tobacco R2R3 MYB-type transcription factor homolog) specifically regulates defense responses in BY-2 cells by enhancing phenylpropanoid metabolism in response to AaGlucan and laminarin elicitors (Shinya *et al.* 2007) and NtChitIV expression is particularly induced by AaGlucan (Shinya *et al.* 2007). A cell wall alpha-glucan from BNR (binucleate Rhizoctonia), induces beta-1,3 glucanase activities in potato sprouts, a primary site of infection by *R. solani* (Wolski *et al.* 2005). Beta-glucan elicitor induced programmed cell death in potato suspension cultures (Mizuno *et al.* 2005), plasma membrane depolarization in soybean roots (Mithofer *et al.* 2005) and the initiation of defense signaling in suspension-cultured cells of *Lycopersicon peruvianum* is induced by the peptide systemin, as well as by chitosan and beta-glucan elicitor from *Phytophthora megasperma* (Stratmann *et al.* 2000). For example, the main effective chemical responsible for paclitaxel formation in fungal endophyte culture supernatant (FECS) is reported to be an exopolysaccharide (EPS) of molecular weight similar to 2 kDa in *Taxus cuspidate* cells (Li *et al.* 2009).

Chitin (β -1,4-linked polymer of N-acetylglucosamine) is an important structural component of fungal cell walls, and is also a well-known oligosaccharide elicitor of immune responses in plants (Kohlmann *et al.* 2015). It also induces nodulation signaling in *Lotus japonicas* (Wang *et al.* 2014). The hyper accumulation of momilactones and phytocassanes due to the hyper-inductive expression of the relevant biosynthetic genes and the MEP pathway gene OsDXS3 in OsTGAP1-overexpressing (OsTGAP1ox) rice cells has previously been shown to be stimulated by the chitin oligosaccharide elicitor (Hayafune *et al.* 2014). Chitin oligosaccharides, chitosan and methyl jasmonate (MJ) stimulate lipoxygenase (LOX) activity in wheat (*Triticum aestivum*) leaves (Bohland *et al.* 1997).

Chitosan, a hydrophilic biopolymer industrially obtained by N-deacetylation of chitin, can be applied as an antimicrobial agent (Rabea *et al.* 2003). Chitosans induce the accumulation of the antifungal phytoalexin pisatin in pea pods and antinutrient proteinase inhibitors in tomato leaves (Walker-Simmons *et al.* 1983). Chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis* (Lee *et al.* 1999). Chitosan also induces the accumulation of phytoalexins in tissues of host plants, decreases the total content and changes the composition of free sterols producing adverse effects on infesters, activated chitinases, beta-glucanases, and lipoxygenases, and stimulates the generation of reactive oxygen species (Vasyukova *et al.* 2001). Barley mildew and its elicitor chitosan promotes closed stomata by stimulating guard-cell S-type anion channels (Koers *et al.* 2011). It has also been

reported that the addition of chitosan, chitosan oligosaccharide and alginate oligosaccharide to the culture of *P. ginseng* hairy roots caused inhibition of growth but total ginseng saponin accumulated slightly with an increase in elicitor concentration (Jeong *et al.* 2005, Jeong *et al.* 2005).

Cyclodextrins are cyclic oligosaccharides that chemically resemble the alkyl-derived pectic oligosaccharide which are naturally released from the cell walls during fungal attack, and they act as true elicitors. When added to plant cell culture, they induce the expression of genes involved in some secondary metabolism pathways. For example, cyclodextrins were shown to enhance the accumulation of trans-resveratrol, one of the basic units of the stilbenes derived from the phenylpropanoid pathway in *Vitis vinifera* suspension cultured cells (Pietrowska-Borek *et al.* 2014). Glycoproteins is one of the microbial cell wall components which can induce HR response. Adding α - or β -glycoprotein from plant pathogenic fungi can generate antibiosis metabolites in lucerne (*Medicago sativa*) suspension culture cells (Walton *et al.* 1993). *Pythium oligandrum* produces glycoprotein elicitor in the cell wall fraction, designates CWP, and induces resistance to a broad range of pathogens (Takahashi *et al.* 2006). A glycoprotein elicitor, CSBI, isolated from hyphal cell walls of the strain 97-151a of a specific glycoprotein elicitor from *Magnaporthe grisea* causes lipid peroxidation and HR reaction in rice leaves which may play an important role in the resistance of rice seedlings (Li *et al.* 2004). A glycoprotein of 34 kDa (GP 34) has been solubilized at acidic pH from the mycelium of *Phytophthora parasitica* var. *nicotianae* (Séjalon-Delmas *et al.* 1997). A fungal glycoprotein elicits cell death, expression of defense genes, production of salicylic acid, and induces systemic acquired resistance in tobacco (Baillieul *et al.* 1995).

1.2 Protein

Fungal protein elicitors which include some enzymes and substances having protein like properties, such as cellulose of *Trichoderma viride* which induces a specific hypersensitive-like response characterized by the formation of resveratrol (3,5,4'-trihydroxystilbene) oxidation products (ROPs). Many proteins derived from fungus can induce hypersensitivity responses and cause the accumulation of secondary metabolites. For example, elicitors derived from *Phytophthora cryptogea* have a protein like property and can also cause hypersensitivity response resulting in the accumulation of metabolites at large. Fungal elicitor protein PebC 1 from *Botrytis cinerea* improves disease resistance in *Arabidopsis thaliana* through the ethylene signal transduction pathway and enhances plant growth, causes drought tolerance and disease resistance in tomato (Zhao *et al.* 2014). A fungal protein elicitor PevD1 induces verticillium wilt resistance in cotton (Bu *et al.* 2013) and a novel protein elicitor (SsCut) from *Sclerotinia sclerotiorum* induces multiple defense responses in the plant (Zhang *et al.* 2014).

The functional domain of enzymes has also been investigated since plant-pathogenic fungi produce cellulases. However, little information is available on cellulase as an elicitor in plant-pathogen interactions. It has been reported that an endocellulase (EG1) isolated from *Rhizoctonia solani* contains a putative protein of 227 amino acids with a signal peptide and a family-45 glycosyl hydrolase domain. Its aspartic acid (Asp) residue at position 32 was changed to alanine (Ala), resulting in full loss of its catalytic activity hence the enzymatic activity of this endoglucanase is not required for its elicitor activity (Ma *et al.* 2015).

1.3 Polyunsaturated fatty acids

Polyunsaturated fatty acids have not been investigated fully as an elicitor to induce resistant

reaction. Polyunsaturated fatty acid (arachidonic acid) and their lipid parts can induce the production of phytoalexin and lignin. Eicosapentaenoic and arachidonic acids in extracts of *Phytophthora infestans* mycelium have been identified as the most active elicitors of sesquiterpenoid phytoalexin accumulation in potato tuber slices (Bostock *et al.* 1982). The activity of lipoxygenase (LOX) in aged potato tuber discs increased by almost 2-fold under the treatment of the discs with the fungal elicitor arachidonic acid (AA) (Bostock *et al.* 1992). The fungal elicitor arachidonic acid can induce cystatin genes in tomato (*Solanum lycopersicum*) using a cDNA expression library from arachidonate-treated leaves (Girard *et al.* 2007).

2. The receptor of elicitor signaling

Signal perception is the first committed step of the elicitor signal transduction pathway and much effort has been put into the isolation of effective elicitor signal molecules from fungal and plant cell extracts or other sources, and in the identification of the corresponding receptors from plant plasma membranes. Receptors are located on the plant cell plasma membrane, and may be encoded by products of plant defense genes or the protein structure of membrane and can be identified and selectively combined with signal molecules. It is now clear that there are several different classes of components that can completely substitute for fungal elicitors in the elicitation effect. These include poly- or oligosaccharides such as chitin, and chitosan and their fragments, xyloglucans, laminarin and other β -glucans and their fragments and oligogalacturonides, proteins or peptides, as well as lipid derivatives such as syringolide, nod factors and lipopolysaccharides. Poly- or oligosaccharides are the most studied signal molecules in elicitor signal transduction. Many elicitors, such as chitin, xyloglucans, chitosan, β -glucan and oligogalacturonide, exhibit elicitor activity across different plant species and significantly induce phytoalexins in plants, suggesting that different plants possess some common receptors to sense these signals. Binding tests with specific saccharide elicitors, using membrane-enriched fractions have led to the discovery of a number of specific receptors. Interaction of most of these saccharides and proteinaceous or lipid elicitors and their receptors show high affinity, specificity, are reversible, and display saturable binding, which indicates they are displaying receptor-ligand interactions (Zhao *et al.* 2005). The search for receptors for glucan oligosaccharide elicitors was initiated by Yoshikawa *et al.* with the use of a radio-labeled elicitor-active polysaccharide, mycolaminaran. The membrane binding sites are mycolaminaran-specific receptors which are physiologically involved in the initiation of phytoalexin production in soybean cotyledons. Because the binding of mycolaminaran to membranes was abolished by heat and proteolytic enzymes, the receptor is probably a protein(s) or glycoprotein(s) (Ben-David *et al.* 1983).

Another type of well-researched receptor is oligoglucoside and there is a direct correlation between the binding affinities and the elicitor activities of these oligoglucosides. Thus, the hepta-beta-glucoside-binding protein fulfills criteria expected of a bona fide receptor for the elicitor-active oligosaccharin (Cheong *et al.* 1991). Beta-glucan elicitor (GE), released from the cell wall of the phytopathogenic fungus *Phytophthora megasperma* by soybean glucanases, causes defense reactions in soybean. A GE-binding protein (GEBP) was purified from the membrane fraction of soybean root cells, and its cDNA was isolated which encodes a GE receptor and may mediate the signaling of the elicitor (Umemoto *et al.* 1997). The beta-glucan-binding protein had an apparent molecular mass of 78 kDa when subjected to SDS-PAGE. The beta-glucan-binding proteins of French bean and soybean are conserved homologs involved in beta-glucan elicitor recognition (Mithöfer *et al.* 1999). The intrinsic endo-1,3-beta-glucanase activity of the GBP is

perfectly suited during initial contact with *Phytophthora* to release oligoglucoside fragments enriched in motifs that constitute ligands for the high affinity binding site present in the same protein (Fliegmann *et al.* 2004). In plants, receptors containing LysM motifs are responsible for the perception of chitin-oligosaccharides. These are involved in beneficial symbiotic interactions between plants and bacteria or fungi, as well as plant defense responses, which can bind to N-acetylglucosamine-containing carbohydrates, such as chitin, chitio-oligosaccharides and peptidoglycan (Akcapinar *et al.* 2015). Although there are few reports on the receptors for fungal elicitors in medicinal plants, the method and theory of receptor research in other species can be used to conduct extensive studies- in medicinal plants.

3. The identification of fungal elicitor

There may be a group or several groups of specific inducer binding sites in medicinal plant cell surface, which can identify and combine elicitors as the first step to induce reactions, however elicitors do not take part in the secondary metabolic processes directly. Previous studies have indicated that receptor proteins display structure specificity, which can identify some specific elicitors and whilst having no effect on other receptors (Barrett *et al.* 2012, Fliegmann *et al.* 2004). There are some reports demonstrating that elicitors are recognized by plant receptors or R proteins localized in the plasma membrane or in the cytoplasm before initiating signaling responses, which (among other responses) lead to elevated production of secondary metabolites (Zhao *et al.* 2005). Legumes endogenous fungal elicitor can identify glucans of many plants. A 1,6-beta-linked and 1,3-beta-branched heptaglucoside (HG), present in cell walls of the oomycetal pathogen *Phytophthora sojae* displays high specificity and affinity towards HG-binding site contained in the beta-glucan-binding protein (GBP) of plasma membrane-enriched fractions. The GBP is composed of two different carbohydrate active protein domains, one containing the beta-glucan-binding site, and the other related to glucan endoglucosidases of fungal origin (Cosio *et al.* 1988, Fliegmann *et al.* 2004, Sacks *et al.* 1995, Schmidt *et al.* 1987). Recently, many studies have been published about protein receptors of endophytic fungal elicitors, such as the protein receptor of chitin which has been investigated in detail and provides full basis of identification mechanism. As researched, various mutualists employ chitin-derived signaling molecules to prepare their hosts for their mutualistic relationship (Kaku *et al.* 2006, Sanchez-Vallet *et al.* 2015) and the first encoding the rice chitin-binding protein (CEBiP) gene was cloned. These authors found CEBiP located on a cell surface and having extracellular LysMs, but lacking a cytoplasmic signaling domain (Kaku *et al.* 2006). As to the identification of CEBiP for chitin, it was reported that CEBiP binds to chitin directly and proposed that two CEBiP monomers bind to the same chitin oligosaccharide from the opposite sides (Hayafune *et al.* 2014). Besides, syringolides, a water-soluble, low-molecular-weight elicitors, and P34, the receptor that mediates syringolide signaling, exhibited ligand-specific ¹²⁵I-syringolide binding activity (Ji *et al.* 2006). Thus fungal elicitors are identified and cause a similar response of producing secondary metabolites in medicinal plants, and the relationship between fungal elicitors and corresponding receptors of medicinal plants deserves further investigation.

4. Signal transduction

Elicitor signal transduction process is a complex network, these signals are integrated into DNA transcription factors, eventually causing defense reactions and triggering metabolic pathways of secondary metabolites (Figure 1). Nitric oxide (NO), salicylic acid (SA), and reactive oxygen species (ROS) are important signal molecules that mediate plant resistance reactions and

play important roles in secondary metabolism (Gao *et al.* 2012). The fungal elicitor-induced secondary metabolites accumulation is one of the defense response behaviors. There are a lot of reports stating that the induction type of plant defense response is dependent on the cross effect between such as SA, JA, ET - signaling pathways to provide one of the best defensive systems (Li *et al.* 2015, Nair *et al.* 2015, Zhu *et al.* 2015), but the integration of different stress signal and the signal pathways interactions of precise regulation mechanism is still unclear. We summarize several signaling pathways like ion fluxes and Ca²⁺ signaling, ROS signaling, JA signaling pathway, SA signaling pathway, NO signaling pathway, other signaling molecules and their crosstalk in the medical plants induced by fungal elicitors is presented in Table 2. The whole process is as shown in Figure 2: Fungal elicitors are identified and combine to receptors selectively on the cell membrane. In the next step regulation of the expression of related genes in the nucleus through complicated signal transduction pathway takes place, and ultimately activates the defensive secondary metabolism system to accumulate specific secondary metabolites. There are many signal molecules involved in the fungal elicitors-induced secondary metabolites in medical plants, such as Ca²⁺, cAMP, inositol phosphate, G protein, salicylic acid, jasmonic acid, NO, ETH and so on. The signal transduction is intricacy and several signal molecules interact and talk with one another under different circumstances. In general, fungal elicitors cause the ion channels to open and G protein coupling through IP₃ as intracellular second messengers. The production of ROS through oxidative burst is common and universal. O₂⁻ and H₂O₂ transform mutually and mediated different signal pathway in various medical plants and NO can respectively delivery the signaling through dependent- or independent- oxidative burst signaling pathway. NO can promote the accumulation of SA and SA can hold up the synthesis of JA in plant cells. There is a relationship of mutual inhibition but also special coordination which is complementary between SA and JA signaling pathways. All those signal molecules are involved in the accumulation of secondary metabolites in fungal-induced medical plants however, the mechanism is still not clearly defined, but they eventually respond to transduction factors which is a bridge between signal molecules and gene expression.

4.1 Ion fluxes and Ca²⁺ signaling

During fungal elicitor treatment electron flow in plant cells takes place in the short term; however the mechanisms are involved are not clearly defined. The early response of plant cells to elicitor stimulation is the flow of ions such as K⁺ / H⁺, Cl⁻ / Ca²⁺ (Almagro *et al.* 2012, Hamada *et al.* 2014) among these the flow of Ca²⁺ ions is a key physiological response (Du *et al.* 2015, Gao *et al.* 2012, Mizuno *et al.* 2005, Zhao *et al.* 2007, Zhao *et al.* 2003).

Perception of elicitor signals activates receptor-coupled effectors, such as GTP-binding proteins (G- proteins) or protein kinases, which further activate ion fluxes (Aharon *et al.* 1998). Among these Ca²⁺ influx and the transient calcium pulse in the cytosol are the most important events in plant defense responses since calcium plays a central role in various signal transduction systems (Keller *et al.* 1998). Ca²⁺ influx is required for elicitor-induced production of beta-thujaplicin in *Cupressus lusitanica* cell cultures and receptor-coupled G-proteins are likely to be involved in the elicitor-induced biosynthesis of beta-thujaplicin. Indeed, both GTP-binding activity and GTPase activity of the plasma membrane are stimulated by elicitor, and suramin and cholera toxin affected G-protein activities, which suggest that Ca²⁺ and G-proteins may mediate elicitor signals to the jasmonate pathway, and the jasmonate signaling pathway may then lead to the production of beta-thujaplicin (Zhao *et al.* 2003). The phosphoinositide degradation is found in

a variety of plants. PLC is activated by Ca^{2+} and IP_3 can transport Ca^{2+} from intracellular stores into the cytoplasm (Aharon *et al.* 1998). Thus, IP_3 - Ca^{2+} signaling pathway is thought to be the main regulatory mechanism of inducing phytoalexin (Altuzar-Molina *et al.* 2011) and cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are involved in anion channel and Ca^{2+} flow, both are important signal channels of accumulation of secondary metabolites in many medical plants (Pietrowska-Borek *et al.* 2014, Wu *et al.* 2008).

Fungal elicitor is recognized by the receptors in the plant, followed with Ca^{2+} burst in many medical plants (Du *et al.* 2015, Genre *et al.* 2013, Lecourieux *et al.* 2002, Zhao *et al.* 2004). Ca^{2+} is a significant second messenger involved in the signal transduction progress of endophytic fungi-induced secondary metabolite accumulation in medical plants (Vasyukova *et al.* 2001). It is well established that the changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ occur in response to a wide variety of physiological responses, however, the distinction of Ca^{2+} signal to distinct endophytic fungal elicitors needs to be addressed. Different frequency and region-specificity of $[\text{Ca}^{2+}]_{\text{cyt}}$ plays a pivotal role in various specific stimulations, including calcium transients, calcium vibration, spatial orientation, and calcium waves. This decides the encoding specificity of Ca^{2+} and the decoding-specificity which follows is also a main step to the different responses, and is recognized and executed by Ca^{2+} sensors which contain two classes. One is calcium dependent protein kinases (CDPK), whose structure domain is combined with calcium ions and protein kinases, such as in CDPK6 mediates stomatal closure induced by yeast elicitor (YEL) (Ye *et al.* 2013); others can produce signal effects by not only combining with Ca^{2+} but also interacting with the target proteins, mainly protein phosphatases. There are calmodulin (CAM) and calcineurin B-like proteins (CBL) already identified in the latter class and they all have EF hand structure domain which can combine with Ca^{2+} . These Ca^{2+} sensors have a decisive role in decoding Ca^{2+} signaling and different Ca^{2+} sensors have different effects depending on the circumstances. For example, CBL is regarded as a protein kinase and its target protein is CBL interacting protein kinase family (CIPK.) There are 10 kinds of CBL and 25 kinds of CIPK identified in *Arabidopsis*. As to the Ca^{2+} signature and decoding system, limited amount of research has been conducted in the process of endophytic fungi-induced accumulation of secondary metabolites in medical plants. The utilized in determining Ca^{2+} signature in crops against fungal pathogens can be used to study its specificity in endophytic fungal-induced accumulation of secondary metabolites.

Ca^{2+} fluxes depend on influx from Ca^{2+} channel and efflux from Ca^{2+} pump. Yeast elicitor induced *Sage Salvia* cell, through the second messenger IP_3 prompts Ca^{2+} channel opening, leading to the enhancement of the activity of phospholipase C, which activates the biosynthetic pathways of polyphosphoinositide sugar leading in the improvement of the quantity of phytoalexin.

4.2 ROS signaling molecule

Reactive oxygen species is a by-product of aerobic metabolism the levels of which are kept in equilibrium in plants under physiological circumstances. If this equilibrium is disturbed this can lead to oxidative stress. Reactive oxygen species, include superoxide anion (O_2^-), hydroxyl free radical ($\cdot\text{OH}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\text{O}_2$), etc, which can transform mutually. These can directly activate the defensive genes and the synthesis of phytoalexin as a second messenger which can form small molecules, such as JA signal. This in return activates and regulates the expression of downstream defense genes as the substrates of lipoxygenase through the lipoxygenase pathway. Reactive oxygen species (ROS) generated in response to stimuli and during development can also function as signaling molecules in eukaryotes, leading to specific

downstream responses. Among these, hydrogen peroxide (H_2O_2) plays an important role as a key signaling molecule in response to various stimuli and is involved in the accumulation of secondary metabolites (Hao *et al.* 2014, Orbán *et al.* 2013, Wang *et al.* 2005, Wang *et al.* 2007) however hydrogen peroxide from the oxidative burst is not always related to secondary metabolite accumulation. For example, the production of H_2O_2 has significant positive correlations with both gene expression and taxanes, indicating that the increase in H_2O_2 might be involved in the upregulation of the taxane production in yew under squalestatin, a fungal metabolite. Hydrogen peroxide from the oxidative burst is not involved in the induction of taxol biosynthesis in *Taxus chinensis* cells (Lan *et al.* 2003). In elicitor-treated soybean cell culture, H_2O_2 mediates the production of isoflavonoid glyceollin. ROS are clearly not involved in the endophytic fungus-host interaction signaling pathway on isoeuphpekinensin accumulation in *Euphorbia pekinensis* suspension cells induced by an endophytic fungal elicitor. Biphenyls are unique phytoalexins produced by plants belonging to *Pyrinae*, a subtribe of the economically important *Rosaceae* family, and aucuparin is a well-known biphenyl. Endogenous generation of H_2O_2 rather than that of O_2^- is a key factor in yeast-induced accumulation of biphenyl phytoalexins in cell cultures of *Sorbus aucuparia* (Qiu *et al.* 2012). Nevertheless, O_2^- rather than H_2O_2 appears to trigger the subsequent reactions during fungal elicitor induced phytoalexin accumulation in cultured parsley cells (Jabs *et al.* 1997). Protein phosphorylation is involved in the signal transduction processes following elicitor recognition by parsley cells (Dietrich *et al.* 1990). In addition, cyclic dipeptides produced by fungus *Eupenicillium brefeldianum* HMP-F96 induced extracellular alkalization and H_2O_2 production in tobacco cell suspensions (Chen *et al.* 2015).

In some plants metabolic induction leads to the generation of ROS which rely mainly on NADPH oxidase and other oxidase and peroxidase present in the mitochondria and chloroplasts. There are some studies demonstrating that NADPH oxidase in plasma membranes is involved in H_2O_2 accumulation in fungal elicitor-induced *Taxus chinensis* cell cultures (Qin *et al.* 2004). Activation of phospholipase C is also involved with the oxidative burst which might constitute one pathway by which elicitors trigger the soybean oxidative burst (Legendre *et al.* 1993). The production of H_2O_2 derived from the plasma-membrane NADPH oxidase and the subsequently increase of cytosolic calcium ions are both required for the activation of the octadecanoid pathway by Pep-25 treatment in *A. thaliana* (Hu *et al.* 2009).

Numerous experiments show the effects of ROS on secondary metabolites production caused by other elicitors rather than fungal elicitors. Ag^+ addition increases H_2O_2 content and phenylalanine ammonia-lyase activity is significantly increased which leads to higher echinacoside and acteoside contents in cell suspension cultures of *Cistanche deserticola* Ma. (Chen *et al.* 2007). SA significantly enhanced H_2O_2 production, phenylalanine ammonia-lyase (PAL) activity, and RA accumulation whilst exogenous H_2O_2 can also promote PAL activity and enhance RA production. If H_2O_2 production is inhibited by NADPH oxidase inhibitor (IMD) or scavenged by quencher (DMTU), RA accumulation will be blocked. These results indicated that H_2O_2 is secondary messenger for signal transduction, which can be induced by SA significantly and promotes RA accumulation (Hao *et al.* 2014). ROS mediated MJ-induced tanshinone production, but SNP-induced tanshinone production was ROS independent in *Salvia miltiorrhiza* hairy roots.

4.3 JA signaling pathway

The phytohormones jasmonates (JAs) not only constitute an important class of elicitors (Walker *et al.* 2002), but also act as a signal molecule for many plant secondary metabolic

pathways(Gundlach *et al.* 1992, Lackman *et al.* 2011, Menke *et al.* 1999).JA and its octadecanoid precursors have also been implicated as intermediate signals in elicitor-induced secondary metabolite accumulation in medicinal plants. In different plant species, secondary metabolite biosynthesis is regulated by the phytohormone jasmonic acid (JA), which is derived by the action of lipoxygenase(Rahimi *et al.* 2014). Cis-jasmonic acid and its precursors tend to be the signaling molecules in the elicitation process(Mueller *et al.* 1993).The integral role of jasmonic acid and its derivatives in the intracellular signal cascade begins with the interaction between an elicitor molecule and the plant cell surface which results ultimately in the accumulation of secondary compounds(Gundlach *et al.* 1992).A significant elevation of intercellular jasmonic acid was observed up to day 6 after elicitation with *A. alternata* (50 μ g l⁻¹) in the whole plant culture of *Solenostemon scutellarioides*, which indicated that JA is involved in this elicitation. It has also been reported that *Aternaria alternata* elicited the biosynthesis of rosmarinic acid via signal transduction through jasmonic acid coupled with elicitor induced oxidative stress and associated mechanism(Dewanjee *et al.* 2014).

JA usually mediates the secondary metabolites synthesis together with other signal molecules and in the progress they play different roles. For instance, both H₂O₂ and JA signals were involved in HEJ-induced taxuyunnanine C (Tc) biosynthesis, and JA mediated the induction of GGPPs (geranylgeranyl diphosphate synthase) and TS (taxa-4(5),11(12)-diene synthase) genes expression, but H₂O₂ was not essential to activate them during enhancing Tc production elicited by a novel synthetic jasmonate in cell cultures of *Taxus chinensis*(Hu *et al.* 2006).Although inducing phytoalexins is one mechanism of defense to fungal elicitors, phytoalexins synthesis and defense systems have their respective signaling pathways. In *Panax ginseng* adventitious roots, mono- and sesquiterpenoid accumulation induced by SA and YE occurs due to the farnesyl diphosphate synthase (FPS) and isopentenyl pyrophosphate isomerase (IPPI) expression and may be mediated by reactive oxygen species signaling and jasmonic acid signal transduction(Rahimi *et al.* 2014).On the other hand, JA signaling pathways sometimes interact with other signaling pathway. For example, phytoalexins are induced in rice infested by *Sogatella furcifera* as antimicrobial compounds mainly through activation of the JA-mediated pathway, but as the defense signal systems, SA- and JA-mediated pathways are activated by the elicitor(Kanno *et al.* 2012).

4.4 SA signaling pathway

Salicylic acid (SA) is induced in plants due to pathogen interaction but is not present in all plants as a defense mechanism(Jayakannan *et al.* 2015). SA is well known to promote some secondary metabolites accumulation as an abiotic elicitor For example SA induced pilocarpine accumulation in *Jaborandi folium*(Avancini *et al.* 2003)and inapic acid, rutin and naringin were detected only in SA treatments to peppermint and antioxidant capacity was also improved(Pérez *et al.* 2014). However, SA can also induce the accumulation of secondary metabolites induced by fungal elicitor. Its role in regulating the secondary metabolite accumulation of plants induced by arbuscular mycorrhizal fungi has also been noted(Zhang *et al.* 2013). For example, the endophytic fungal elicitor induced increased NO content and SA production, which promoted isoeuphpekinensin accumulation in *Euphorbia pekinensis* suspension cells(Gao *et al.* 2011).

4.5 NO signaling pathway

Secondary metabolite accumulation and nitric oxide (NO) generation are two common responses of plant cells induced by fungal elicitors, and NO has been reported to play important roles in elicitor-induced secondary metabolite accumulation(Lu *et al.* 2011). Nitric oxide (NO) is a

bioactive molecule that exerts a number of diverse signal functions in phylogenetically distant species (Beligni *et al.* 2000). Crude elicitor of one endophytic fungi (belong to *Cunninghamella sp.*, named AL4) induced multiple responses in *Atractylodes lancea* suspension cells, including rapid generation of nitric oxide (NO) and hydrogen peroxide (H₂O₂), sequentially followed by enhancement of essential oil production. NO and H₂O₂ may all act as signaling molecule to mediate AL4 elicitor promoting essential oil accumulation in suspension cells of *A. lancea*, but AL4 elicitor can also promote essential oil accumulation in suspension cells of *A. lancea* by other mechanisms (Fang *et al.* 2009). Maojun Xu regards NO as the key node of complicated transduction network, which may act as potential molecular switch in the process of fungal-induced secondary metabolites accumulation.

Fungal can induce the production of NO by nitric oxide synthase (NOS), nitrate reductase (NR) and inorganic nitrogen in medical plants jointly. The response of soybean cotyledons to the fungus *Diaporthe phaseolorum f. sp. meridionalis* (Dpm) elicitor involves NO formation via a NOS-like enzyme that triggers the biosynthesis of antimicrobial flavonoids (Modolo *et al.* 2002). NR is involved in the fungal elicitor-triggered NO generation and the fungal elicitor induces camptothecin production of *Camptotheca acuminata* cells dependently on NR-mediated NO generation (Lu *et al.* 2011). The current work aimed to characterize the generation of nitric oxide (NO) and gene expression of luteolosynthase (LUS) which is a crucial enzyme regulating the synthesis of luteolin in *Betula platyphylla* cells exposed to a *Phomopsis* elicitor. LUS gene expression was found to increase abruptly 10 h after *Phomopsis* induction, reaching its peak level (18.08) at 24 h. The effects of nitrate reductase (NR) and NO synthase (NOS), the two key enzymes responsible for endogenous NO biosynthesis in plants, have also been investigated. NO production in *B. platyphylla* cell cultures exhibited a biphasic pattern, reaching the first plateau within 1.0-10 h of exposure to the *Phomopsis* elicitor. The maximum levels of NOS and NR activities in elicitor-treated cells were found to be 1.7-fold and 6.9-fold those of untreated cells, respectively. Pharmacological experiments showed that *Phomopsis* elicitor-induced NO production and LUS gene expression level were significantly suppressed by the NOS inhibitor N-G-nitro-L-Arg methyl ester (L-NAME), the NR inhibitor sodium azide (NaN₃), and the NO-specific scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO). NaNO₂ and L-arginine (the substrates that produce NO via NR and NOS) and NO donor sodium nitroprusside (SNP) were found to increase both NO production and LUS gene expression. These results suggest that the increase in LUS gene expression due to fungal elicitor-induced NO may involve the NR and NOS biosynthetic pathways (Fan *et al.* 2013). Similarly elicitor prepared from the cell walls of *Penicillium citrinum* induced multiple responses in *Catharanthus roseus* suspension cells, including rapid generation of nitric oxide (NO), sequentially followed by enhancement of catharanthine production by *C. roseus* cells. Elicitor-induced catharanthine biosynthesis was blocked by NO-specific scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide and nitric oxide synthase (NOS) inhibitor S,S'-1,3-phenylene-bis(1,2-ethanediy)-bis-isothiourea (PBITU) which also strongly inhibited elicitor-induced NO generation by *C. roseus* suspension cells. The inhibiting effect of PBITU on elicitor-induced catharanthine production was reversed by external application of NO via the NO-donor sodium nitroprusside. These results strongly suggested that NO, generated by NOS or NOS-like enzymes in *C. roseus* suspension cells when treated with the fungal elicitor, was essential for triggering catharanthine synthesis (Xu *et al.* 2006).

4.6 Other signaling molecules

There are some other molecules involved in the secondary metabolites induced by fungal elicitors, such as ETH, ABA and inositol 1,4,5-trisphosphate(IP₃), but studies on these are rather limited and they have little influence on the fungal elicitation in medicinal plants.

First, ethylene is necessary and specific for the secondary metabolites accumulation of several medicinal plants. For instance, *Amaranthus mangostanus* seedlings treated with ethylene as 5 mM ethphon also had elevated levels of betacyanin. In contrast, salicylic acid (SA) and H₂O₂ treatments had no influence on betacyanin contents in light or dark environment (Cao *et al.* 2012). Secondly, ABA is also a significant signaling molecule in the accumulation of secondary metabolites. For example, treatment of suspension cells of *Ginkgo biloba* with fungal endophytes resulted in accumulation of flavonoids, increases abscisic acid (ABA) production and activation of phenylalanine ammonia-lyase (PAL). It can be concluded that there is a causal relationship between ABA release and both PAL activity and flavonoid accumulation under fungal endophytes treatment. Thus, ABA is involved in fungal endophytes-induced flavonoids accumulation in this plant (Hao *et al.* 2010). However, ABA shows specificity in the kinds of secondary metabolites for example, ABA is not required for the induction of capsidiol synthesis but is essentially implicated in a stress-response checkpoint to fine-tune the amplification of capsidiol synthesis in elicitor-induced wild tobacco (Mialoundama *et al.* 2009). Thirdly, there are some kind of relationships between the phosphoinositide pathway and the accumulation of secondary metabolites, but this is not well-researched in the area of fungal-induced secondary metabolites production of medicinal plants. As researched, the activation of phosphatidylinositol 3-kinase (PI3K) causes the release of phosphoinositide and diglyceride (DG), both of which are induced respectively by different biochemical pathways. Phosphate inositol opens the Ca²⁺ channel by activating the IP₃ of endoplasmic reticulum, while DG generates diacylglycerol which combines with protein kinase and activates protein kinase C to trigger protein phosphorylation signaling cascade. There are reports showing IP₃ pathway starting with activation of PLC by a receptor-coupled protein, generally a GTP-binding protein in animals, and with hydrolysis of phosphatidylinositol 4,5-bisphosphate (Zhao *et al.* 2004). Many of the plant response to fungal-stimuli are related with PLC- inositol phospholipids pathway. For instance, elicitor treatment also doubled phospholipase A₂ activity and the simultaneous treatment of aristolochic acid. A phospholipase A₂ inhibitor, inhibited triterpenoids accumulation as well as phospholipase A₂ activity in *Scutellaria baicalensis* suspension cultures using a yeast elicitor (Yoon *et al.* 2000) and IP₃ rapidly accumulates in yeast elicitor-treated *Cupressus lusitanica* Mill. cells by 4- to 5-fold over the control.

4.7 The crosstalk between signaling

Although there are many types of induction signals, only specific signals are involved in a particular induced reaction. Multiple signaling pathways function not singly but form a crisscross network, which finally produces specific substance metabolism synthesis through mutual coordination. Cross-talk between signal transduction pathways is a central feature of the tightly regulated plant defense signaling network (Li *et al.* 2006).

There is a copper-induced cross talk among calcium, H₂O₂, NO and a calcium-dependent activation of gene expression involving calmodulins and calcium-dependent protein kinases (González *et al.* 2012). The regulation of tobacco (*Nicotiana tabacum*) alkaloid biosynthesis is regulated by JA and abscisic acid (ABA) signaling pathway, and a tobacco gene from the PYR/PYL/RCAR family, NtPYL4, the expression of which is regulated by JAs, was

found to encode a functional ABA receptor(Lackman *et al.* 2011).NO and H₂O₂ induced by oligochitosan lead to a reduction in stomatal aperture and LEA protein gene expression of leaves of *B. napus* L(Li *et al.* 2009). Otherwise, G-proteins and Ca²⁺ are regarded as the transducer of downstream, such as JA signaling during fungal elicitor- induced secondary metabolites accumulation. For example, they are suggested to mediate the elicitor signal transduction to the jasmonate signaling pathway, which is further involved in elicitor- induced production of β-thujaplicin.

NO can act on the upstream of SA, JA, ROS signaling molecules and regulate the formation of SA.NO can stimulate the accumulation of SA like ROS(Durner *et al.* 1998)and SA can also induce the generation of ROS and NO. SA and JA signal pathways not only restrain each other but also have a special coordination complementary action mutually. There is a causal relationship between elicitor-induced NO generation, JA biosynthesis, and hypericin production in *Hypericum perforatum* cells and indicate a sequence of signaling events from NO to hypericin production, within which NO mediates the elicitor-induced hypericin biosynthesis at least partially via a JA-dependent signaling pathway. This declared NO plays a regulatory role in the upstream of JA signaling pathway(Xu *et al.* 2006). However, NO is not fully dependent on JA signaling pathway as NO also mediates an elicitor-induced increase in production of antioxidant polyphenols in *L. obliquus* via a signaling pathway independent of oxylipins or JA, a mechanism which differs from those in some higher plants(Zheng *et al.* 2009). Whether these pathways are JA-dependent or not, which may depend on the plant species. Multiple responses of *Shiraiabam busicola*, including nitric oxide (NO) generation, hypocrellins production and salicylic acid (SA) biosynthesis were induced by a fungal elicitor prepared from the mycelium of *Aspergillum niger*. NO donator, sodium nitroprusside, and SA enhanced hypocrellin production without the fungal elicitor. However, the NO scavenger, 2, 4-carboxyphenyl-4,4, 5,5- tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) and the SA biosynthesis inhibitor, cinnamic acid (CA), inhibited hypocrellin accumulation in the presence of the elicitor. cPTIO also inhibited SA production induced by the *A. niger* elicitor. CA failed to inhibit NO production but it significantly inhibited hypocrellin accumulationthis suggests that NO is at the upstream of SA pathway in mediating the hypocrellin synthesis. The *Aspergillum niger* elicitor induced an NO burst, SA accumulation, and hypocrellin production in *Shiraiabam busicola*. Therefore, the fungal elicitor was involved in the signaling pathway, which is a mechanism different from that of higher plants(Du *et al.* 2015).*Pueraria thomsonii* Benth. cells were treated with the elicitors prepared from cell walls of *Penicillium citrinum* and detected the contents of NO, salicylic acid (SA), jasmonic acid (JA), and puerarin. The results showed that the fungal elicitor induced NO burst, SA accumulation and puerarin production in the cell of *P. thomsonii* Benth. The elicitor-induced SA accumulation and puerarin production was suppressed by nitric oxide specific scavenger cPITO, indicating that NO was essential for elicitor-induced SA and puerarin biosynthesis in the cell of *P. thomsonii* Benth. In the cell of transgenic NahGP. *Thomsonii* Benth., the fungal elicitor also induced puerarin biosynthesis, NO burst, and JA accumulation, though the SA biosynthesis was impaired. The elicitor-induced JA accumulation in transgenic cells was blocked by cPITO, which suggests that JA acted downstream of NO and its biosynthesis was controlled by NO. External application of NO via its donor sodium nitroprusside (SNP) enhanced puerarin biosynthesis in transgenic cell of NahG *P. thomsonii* Benth., and the NO-triggered puerarin biosynthesis was suppressed by JA inhibitors IBU and NDGA, which indicates that the NO induced puerarin production through a

JA-dependent signal pathway in the transgenic cells. Exogenous application of SA suppressed the elicitor-induced JA biosynthesis and reversed the inhibition of IBU and NDGA on elicitor-induced puerarin accumulation in transgenic cells, which indicated that SA inhibited JA biosynthesis in the cells and that SA might be used as a substitute for JA to mediate the elicitor- and NO-induced puerarin biosynthesis. It was, therefore, concluded that NO might mediate the elicitor-induced puerarin biosynthesis through SA- and JA-dependent signal pathways in the cell of wild type *P. thomsonii* Benth. and transgenic NahG cells respectively(Xu *et al.* 2006).

The antagonistic action between jasmonic acid (JA) and salicylic acid (SA) in plant defense responses has been well documented. However, their relationship in secondary metabolite production is largely unknown. A protein elicitor PB90 from *Phytophthora boehmeriae* triggers JA generation, SA accumulation and flavonol glycoside production of Ginkgo biloba cells. JA inhibitors suppress not only PB90-triggered JA generation, but also the elicitor-induced flavonol glycoside production. However, the elicitor can still enhance flavonol glycoside production even though the JA generation is totally inhibited. Over-expression of SA hydrolase gene NahG not only abolishes SA accumulation, but also suppresses the elicitor-induced flavonol glycoside production when JA signaling is inhibited. Interestingly, expression of NahG does not inhibit the elicitor-induced flavonol glycoside accumulation in the absence of JA inhibitors. Moreover, JA levels are significantly enhanced when SA accumulation is impaired in the transgenic cells. Together, the data suggests that both JA and SA are involved in PB90-induced flavonol glycoside production. Furthermore, it has been demonstrated that JA signaling might be enhanced to substitute for SA to mediate the elicitor-induced flavonol glycoside accumulation when SA signaling is impaired, which reveals an unusual complementary relationship between JA and SA in mediating plant secondary metabolite production(Xu *et al.* 2009).

The above illustrates that different signal transduction pathways in cells can inter cross one another, forming a mutually restricted and coordinating signal introduction network in the process of plant secondary metabolism.

5. The integration of the signal by transcription factor

The mechanism of how to integrate different signaling pathways into the synthesis of a single substance is not very clear, but the activation of inducer defense gene expression is dependent on the transcription factors. Transcription factors are DNA binding proteins combining specifically with cis-elements of promoter region in eukaryotic gene. From the theoretical aspect, the regulation of transcription factors can activate the synergic expression of multiple genes and activate the expression of genes regulating similar secondary metabolites synthesis in medical plants(Aharoni *et al.* 2011). The interaction between transcription factors and promoter element or several transcription factors decide the specificity and efficiency of space and induce gene expression to a great extent. For example CrWEKY1 is a transcription factor related to the synthesis of terpenoid in *Catharanthus roseus*, which belongs to WRKY family and CrWEKY1 can express prior to being induced by JA, GA and ETH. CrWEKY1 is one of TIA synthesis network and regulates several key enzymes and transcription factors in terpenoid indole alkaloids (TIA) synthesis pathway. Besides, CrWEKY1 can interact with DXS and SLS during the synthesis of terpenoid(Suttipanta *et al.* 2011). JAs and other signaling molecules can regulate various transcription factors and further regulate the expression of secondary metabolites. For instance, WRKY70 as a node of convergence integrates salicylic acid (SA)- and jasmonic acid (JA)-mediated signaling ev

ents during plant response to bacterial pathogens.(Li *et al.* 2006) Multiple signaling molecules regulate the expression of secondary metabolism synthesis gene by several transcription factors. The study on the relationship between transcription factors and various signaling molecular is relatively scarce during the accumulation of secondary metabolites induced by fungal elicitors in medical plants.

6. Gene expression

Fungal elicitors induce the accumulation of secondary metabolites in medical plants through signal transduction to activate related genes. Fungal- induced signal transmission may induce the synthesis of new enzymes and activate new metabolic pathways to benefit to the synthesis of secondary metabolites probably by increasing the amount of key enzymes involved in the synthesis of secondary metabolites. The fungal elicitors, a xylanase from *Trichoderma viride* and an extract from the cell wall of *Phytophthora infestans*, are shown to cause a rapid reduction in the mRNA levels of various cell cycle-related genes, including MAP kinase genes and cyclin genes, in cultured tobacco cells (*Nicotiana glauca* cv. Xanthi, line XD6S)(Suzuki *et al.* 2007).

Elicitor-induced sanguinarine accumulation in opium poppy (*Papaver somniferum*) cell cultures provides a responsive model system to profile modulations in gene transcripts and metabolites related to alkaloid biosynthesis. Principle component analysis revealed a significant and dynamic separation in the metabolome, represented by 992 independent detected analytes, in response to elicitor treatment. Identified metabolites included sanguinarine, dihydrosanguinarine, and the methoxylated derivatives dihydrochelirubine and chelirubine, and the alkaloid pathway intermediates N-methylcoclaurine, N-methylstylophine, and protopine. Some of the detected analytes showed temporal changes in abundance consistent with modulations in the profiles of alkaloid biosynthetic gene transcripts(Zulak *et al.* 2007). The majority of up-regulated genes are predicted to have a role in the defense response as signaling components and transcription factors as well as the metabolism involved in the production of secondary signaling molecules and antimicrobial compounds after application of two different microbial elicitors, PiE (an elicitor from the cell walls of an oomycete, *Phytophthora infestans*) and TvX (a xylanase from a fungus, *Trichoderma viride*), in tobacco cultured cells. The gene transcription of geranylgeranyl diphosphate synthase (GGPPs) and taxadiene synthase (TS) was up-regulated by HEJ elicitation compared to control in cell cultures of *Taxus chinensis*(Singkaravanit *et al.* 2010).A significant amount of work has been carried out on the gene expression effects of fungal elicitor in hairy roots of medical plants. For example the effects of fungal elicitor artemisinin production were positively correlated with regulatory genes of MVA, MEP, and artemisinin biosynthetic pathways, viz. hmgr, ads, cyp71av1, aldh1, dxs, dxr, and dbr2 in hairy root cultures of *A. annua* L.(Du *et al.* 2015).The enhancing effects of fungal elicitors on lignans production in hairy root cultures of *Linum album* was correlated with the increased expression of some key genes involved in the biosynthesis of these compounds, phenylalanine ammonia-lyase, cinnamoyl-CoA reductase, cinnamyl-alcohol dehydrogenase and pinosresinol-lariciresinol reductase(Wang *et al.* 2005). There are also some reports displaying that fungal elicitors promote the secondary metabolites synthesis, which also causes the change in metabolism genes. Elicitor-induced sanguinarine accumulation in opium poppy (*Papaver somniferum*) cell cultures provides a responsive model system to profile modulations in gene transcripts and metabolites related to alkaloid biosynthesis. An annotated expressed sequence tag (EST) database was assembled from 10,224 random clones isolated from an elicitor-treated opium poppy cell culture cDNA library. The most abundant ESTs

encoded defense proteins, and enzymes involved in alkaloid metabolism and S-adenosylmethionine-dependent methyl transfer. ESTs corresponding to 40 enzymes involved in the conversion of sucrose to sanguinarine have been identified. A corresponding DNA microarray was probed with RNA from cell cultures collected at various time-points after elicitor treatment, and compared with RNA from control cells. Several diverse transcript populations were coordinately induced, with alkaloid biosynthetic enzyme and defense protein transcripts displaying the most rapid and substantial increases. In addition to all known sanguinarine biosynthetic gene transcripts, mRNAs encoding several upstream primary metabolic enzymes were coordinately induced. Fourier transform-ion cyclotron resonance-mass spectrometry was used to characterize the metabolite profiles of control and elicitor-treated cell cultures. Principle component analysis revealed a significant and dynamic separation in the metabolome, represented by 992 independent detected analytes, in response to elicitor treatment. Identified metabolites included sanguinarine, dihydrosanguinarine, and the methoxylated derivatives dihydrochelirubine and chelirubine, and the alkaloid pathway intermediates N-methylcoclaurine, N-methylstylopine, and protopine. Some of the detected analytes showed temporal changes in abundance consistent with modulations in the profiles of alkaloid biosynthetic gene transcripts (Zulak *et al.* 2007).

7. The activation of key enzyme

The proteins are up-regulated and the key enzymes are activated in fungal elicitor-induced secondary metabolites accumulation; with the activation of key enzyme being triggered by key gene expression in a cascade mechanism. Exogenous putrescine enhanced cell viability and antioxidant enzyme activity markedly in cell suspension culture of *Cistanche deserticola*, resulting in an increase in the echinacoside and acteoside production (Chen *et al.* 2007). Cultures treated with chitosan contained 72 proteins when compared to the untreated controls whereas 27 proteins found in controls were not detected in chitosan-treated Moss Plants (*Physcomitrella patens*) cultures. Exopolysaccharide (EPS) and water-extracted mycelial polysaccharide (WOS) from the endophytic fungus *Fusarium oxysporum* Dzf17 significantly increased the activities of phenylalanine ammonia lyase (PAL), polyphenoloxidase (PPO) and peroxidase (POD), suggesting that the oligosaccharides from the endophytic fungus *F. oxysporum* Dzf17 may be related to the activation and enhancement of the defensive mechanisms of *D. zingiberensis* suspension cell and seedling cultures (Li *et al.* 2011). After methyl jasmonate (MeJA) treatment, which is considered an elicitor of secondary metabolites, 388 candidate MeJA-responsive unique transcripts were identified in Sweet Basil (*Ocimum basilicum*). Transcript analysis suggests that in addition to controlling its own biosynthesis and stress responses, MeJA up-regulates transcripts of the various secondary metabolic pathways, including terpenoids and phenylpropanoids flavonoids. Furthermore, combined transcript and metabolite analysis revealed MeJA-induced biosynthesis of the medicinally important ursane-type and oleanane-type pentacyclitriterpenes. Two MeJA-responsive oxidosqualenecyclases (ObAS1 and ObAS2) that encode for 761- and 765-amino acid proteins, respectively, were identified and characterized. Functional expressions of ObAS1 and ObAS2 in *Saccharomyces cerevisiae* led to the production of beta-amyrin and alpha-amyrin, the direct precursors of oleanane-type and ursane-type pentacyclic triterpenes, respectively. ObAS1 was identified as a beta-amyrin synthase, whereas ObAS2 was a mixed amyrin synthase that produced both alpha-amyrin and beta-amyrin but had a product preference for alpha-amyrin. Moreover, transcript and metabolite analysis shed light on the spatiotemporal regulation of pentacyclic triterpene biosynthesis in sweet basil (Misra *et al.* 2014).

8. Application of fungal elicitor for drug production

Compared with other elicitors, fungal elicitor is more effective on the induction of secondary metabolites in most instances (Mandujano-Chavez *et al.* 2000) (Orbán *et al.* 2008) with a few exceptions (Kim *et al.* 2014). For example, the maximum enhancement of artemisinin was achieved with *P. indica* and the accumulation of psoralen increased by the use of fungal elicitor (Ahlawat *et al.* 2014, Ahmed *et al.* 2014). In addition, there are other reports demonstrating that the fungal elicitors are more effective than chemical elicitors under general conditions, but similar to the bacterial elicitors (Awad *et al.* 2014). Microbial elicitation is two-fold higher than commonly used elicitor, methyl jasmonate in root cultures of *Taverniera cuneifolia* (Roth) Arn. for elevated glycyrrhizic acid production. In a few studies, MeJA was consistently found to favor the earlier metabolite (solavetivone), while fungal elicitation promoted conversion to subsequent metabolites in the pathway. Comparatively speaking, some researches reveal that fungal elicitor could substantially improve the total content of volatile oil, while the fungus could more effectively enhance the quality of herbal medicines on *Atractylodes lancea* plantlets.

The fungal elicitor mainly has the following main features in the cultures of plant tissue and cell: The first one is specificity—the same fungal elicitors induce different effects on different plants. The second is rapid response to fungal elicitors, which can usually stimulate the defensive response and cause the accumulation of secondary metabolites in a few hours or days; The concentration effect is the third character and there are two types in the effect of fungal concentration. One of the types is saturated reaction which is that the synthesis of secondary metabolites rises as the fungal concentration is increased continually and the synthesis is maintained at maximum level. While another one is the optimum concentration, which is that the production of secondary metabolites is upmost at the optimum concentration of fungal elicitors. And the fourth is the effect of time. As studied, growth period of plant cells is separated into four stages: delay stage, logarithmic stage, stationary stage and decline stage. The sensibility of fungal elicitors is different to the medical plant cells in different stages and the terminal logarithmic stage and the early stationary stage are the most effective in the accumulation of secondary metabolites added fungal elicitors. The last but not the least, there is synergistic effect between different elicitors, which can promote the synthesis of secondary metabolites (Salas-Marina *et al.* 2011).

An inducer can promote a lot of ingredients in one plant, but the effect on various secondary metabolites is different (Table 1) (Akcapinar *et al.* 2015) (Akcapinar *et al.* 2015). Different elicitors derived from one fungal have different effects on promoting secondary metabolites in medical plants (Li *et al.* 2011), so it's necessary to find out the most effective elicitor. However, addition of fungal elicitor for significantly stimulating secondary metabolites accumulation in the cell culture of *Calendula officinalis* and its secretion into the culture medium, the elicitors also caused slight inhibition of *Calendula officinalis* cell growth (Wiktorowska *et al.* 2010). Thus, we should pay attention to the balance of cell growth and secondary metabolites' product in the large-scale production.

9. CONCLUDING REMARKS

The accumulation of secondary metabolites induced by fungal elicitors can provide new ideas for development and utilization of secondary metabolite and promote the development of medicinal plant. As we all know, the most effective components of medical plants are secondary metabolites and elicitation of plant cells in culture represents a useful biotechnological tool to improve the production of these valuable metabolites. Therefore, the application of fungal elicitors largely

solve the problem of low amounts of the active ingredients such as, the application of squalenol significantly increases taxol and baccatin III yields in cell suspension culture of yew (*Taxus baccata* L.) (Amini *et al.* 2014). However, not all the fungal are useful and suited to the production of secondary metabolites, some pathogenic fungal cause cell hypersensitive responses and programmed cell death, but non-pathogenic fungal may either cause general defense responses or no response. For example, there are some reports displaying that *Pythium* genus, *Pythium irregulare*, *Pythium aphanidermatum* and *Pythium vexans* showed great stimulating activity on alkaloid production (DiCosmo *et al.* 1987), while genus *Mucor* (*Mucor fragilis* and *Mucor rouxianus*) showed a weak elicitor activity. Besides, different fungal elicitors have different effects on the accumulation of secondary metabolites in medical plants (Ahlawat *et al.* 2014). Also, the effects of fungal elicitors are related to a variety of factors such as the subcategories, concentration of elicitors, adding time and the length of inducing time, which is complicated in the application of fungal elicitors. Thus, we should pay more attention to the characters of fungal elicitation for the improvement of application.

It is reported that combined elicitors are most efficacious in the application of fungal elicitor in medical plants, as evident in various reports. Due to different action of different elicitors, the combination of different elicitors may achieve a synergic effect. There are some large-scale industrial productions of secondary metabolites in some medical plants. Fungal elicitors prove beneficial towards modifying both the terpenoids profile and improvement in the composition of essential oil which has important applications for the large-scale production of essential oils and forest biotechnology with respect to spearmint. As to the time and concentration of elicitation, different secondary metabolites in medical plants have different parameters, but this needs a series of experiments to prove it.

The research of plant signal transduction has made important progress in recent years, which can provide the outline of a transduction pathway. However, there are a lot of details which have been not studied clearly in the process of fungal elicitor induced pathways involved in medical plants. However, the signal transduction development of model plant *Arabidopsis thaliana* and model medical plant *Salvia miltiorrhiza* are attracting extensive attention, and we can use them for reference to determine the mechanism of fungal elicitor-induced secondary metabolites' accumulation in medical plants. On one hand, it's necessary to identify the known signal molecules clearly and discover new plant transduction pathways in medical plants. We should give more attention to the relationship between signal system and their time - space characters in fungal elicitor-induced accumulation of secondary metabolites in medical plants. Besides, ABA, Ca²⁺, cAMP, ETH, G protein, IP₃, O₂⁻, ROS and singlet oxygen are less studied in fungal- induced secondary metabolites as outlined in Figure 1b. Therefore, we can focus on the study of these signal molecules for improving the whole lines of signal transduction induced by fungal elicitors in medical plants. On the other hand, the purification of and structure analysis of elicitors, extraction and purification of corresponding receptors, and their combination on cell membrane, needs to be conducted. It is feasible and suitable to adopt new research techniques and approaches, such as gene engineering and research methods, and micro-injection signal transduction molecules diameter, to conform the role of several signaling molecules in regulating gene expression of secondary metabolites synthesis. The methods of defense resistant in crops are relatively mature, and we can follow the steps of defense resistant to study the mechanism from the levels of signal transduction, transcription factors, gene expression and enzyme activation further, laying

foundation for mass production of active substances in medical plants induced by fungal elicitors.

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