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HABILITATION THESIS

**Molecular Aspects of MAMP (Microbe-Associated
Molecular Pattern) Triggered Immunity in Plants**

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Brno 2016

ACKNOWLEDGEMENTS

On the first place I would like to gratefully and sincerely thank deceased prof. Vladimír Mikeš for his guidance, understanding, and most importantly, his friendship and support during my graduate and post-graduate studies in the Department of Biochemistry. His mentorship was essential for my further scientific work and carrier. I would also like to thank all members of the Department of Biochemistry for their patience and all the stimulating discussions we have had.

I would also like to thank Michel Ponchet from the INRA Sophia Antipolis for his assistance and guidance in getting my post-graduate career started on the right foot and for many inspirational discussions about the fascinating world of plant defence, science and life. I also wish to thank Harald Keller from the INRA Sophia Antipolis for many valuable advices in getting my graduate career. I am grateful to Francois Simon-Plas and Nathalie Leborgne-Castel from the INRA Dijon, for introducing me into the field of membrane rafts.

My thanks go to all my colleagues and students for their inspiring thoughts, for excellent work and cooperation and for creation of a stimulative working environment.

Finally, and most importantly, I would like to thank my wife Gabriela for her support, encouragement, quiet patience and unwavering love, my daughters Dominika and Valerie for being a source of motivation and pleasure.

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PREFACE

The present habilitation thesis is a comment summary of original scientific articles published between the years 2005 and 2016 to which I have contributed as the first author, corresponding author or co-author. All these publications are related to the characterization of molecular mechanisms responsible for the defence reaction in plants triggered by Microbial-Associated Molecular Patterns (MAMPs). The text part of the thesis is divided into four main chapters. The first chapter presents a current concept of plant defence mechanisms in plants, covering the generally accepted zig-zag model. The second chapter is dedicated to the description of processes included in MAMPs-Triggered Immunity (MTI) which affords a basic effective level of the resistance to plants. The third chapter describes in detail molecular processes of ergosterol perception which represents an orphan fungal MAMP. The last chapter presents a complexity of factors taking the role in perception of elicitors, a typical oomycetes MAMP, by plants.

A comprehensive information on the studied plant-pathogen interactions can be found in the supplement, together with the list of publications included in this habilitation thesis. At the end of each third and fourth part a contribution to the given topic is presented and in conclusion future prospects of the research are outlined.

1. PLANT DEFENCE MECHANISMS

In their natural environment, plants serve as a rich source of nutrients and that is why they host a great number of non-pathogenic and pathogenic microorganisms, especially on their root system and shoots (leaves, reproductive structures, stem). In contrast to vertebrates, plants are sessile organisms lacking neither specialized immune cells nor an adaptive immune system against continuous attack of a vast array of pathogenic microorganisms such as fungi, oomycetes, viruses, bacteria or nematodes (Jones and Dangl, 2006). To deal with this challenge, plants are protected by mechanical barriers such as a waxy cuticle or protective periderm and they have evolved a multi-layered immune system, in which each cell has a potential capacity to trigger an immune response against pathogen infection. This process called innate immunity is fundamental for survival, and it allows a plant to ward off pathogens both, in a rapid and localized manner, and remember any previous infection (Reimer-Michalski and Conrath, 2016).

Plant pathogens use diverse strategies to colonise their hosts. Pathogenic bacteria enter the plant through pores (stomata or hydathodes) or wounds and proliferate in the intercellular space; nematodes and aphids directly insert a stylet into a plant cell and pathogenic fungi or oomycetes are able to invaginate haustoria into the host cell plasma membrane (Jones and Dangl, 2006). One of the common features of all these diverse pathogen classes is the delivery of some type of effector molecules (virulence factors) into the host cell to enhance microbial fitness (Cui et al., 2015; Reimer-Michalski and Conrath, 2016).

1.1 PLANT-PATHOGEN INTERACTIONS

In general, the plant-pathogen interactions are either incompatible or compatible. The compatible interaction (between a susceptible host and a virulent pathogen) results in a successful pathogen infection and outbreak of the disease and the incompatible interaction results in triggering of a defence response and elimination of the pathogen. In the 1940s, Harold H. Flor proposed the gene-for-gene model to describe the interaction between a pathogen and its host plant (Flor, 1971). This model predicted that a plant resistant phenotype would occur only in that situation when the plant possessed a dominant resistance gene (*R*) and the pathogen expressed a complementary dominant avirulence gene (*Avr*). Even though the model holds true for most biotrophic plant-pathogen interactions, for many other pathogens an indirect activation of R proteins by pathogen encoded-effectors was described. This fact implies that the R proteins are able to recognise indirectly pathogen effectors by monitoring the integrity of host cellular targets of effector action, which is similar to the recognition of “modified self” in “danger signal” models of the mammalian immune system. Based on this knowledge, a new four-phased “zig-zag” model was proposed (Jones and Dangl, 2006).

1.2 ZIG-ZAG MODLE OF PLANT IMMUNITY

This model describes, in essence, two branches of the plant immune system. One uses surface pattern recognition receptors (PRRs) acting as initial radars that recognise slowly evolving microbial-associated molecular patterns (MAMPs) to induce a basal resistance response called PRR-triggered immunity (PTI) (Figure 1) (Böhm et al., 2014; Jones and Dangl, 2006; Zipfel, 2008). PTI is considered as an ancient form of plant immunity and

has the potential to fend off host non-adapted pathogens due to the conserved nature of MAMPs (bacterial flagellin, chitin or oligogalacturonides) across species (Reimer-Michalski and Conrath, 2016). The second takes a place largely inside the cells employing a family of polymorphic intracellular nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins to perceive secreted virulence effectors (previously called Avr protein) directly or indirectly as a result of host-pathogen co-evolutionary cycle (Cui et al., 2015; Jones and Dangl, 2006). The direct interaction of the pathogen effector with plant R proteins leads to gene-for-gene immunity. The indirect recognition of pathogen effector by plant R proteins has been described in the so-called guard hypothesis in which R proteins guard the integrity of cellular proteins and when there is some modification/degradation of proteins by an appropriate effector, they will trigger plant defence (Jones and Dangl, 2006; Reimer-Michalski and Conrath, 2016). Independent of whether pathogen effector is recognised directly or indirectly, its perception results in the effector-triggered immunity (ETI) amplifying PTI response and resulting in a resistance, and usually, a hypersensitive cell death response at the infection site to avoid spreading of biotrophic pathogens to healthy tissue of plants (Figure 1) (Reimer-Michalski and Conrath, 2016).

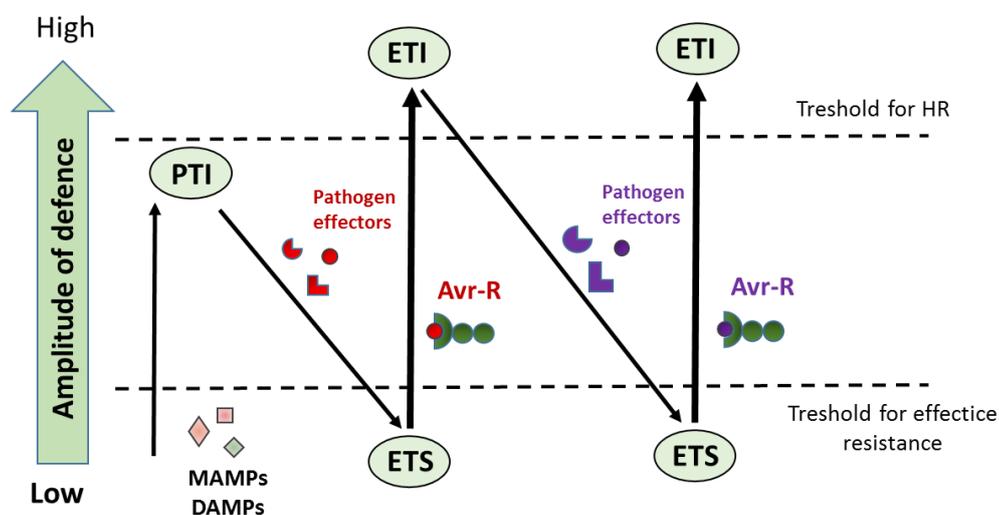


Figure 1. *The Zig-Zag model for evolution of innate immunity- and silencing-based plant defence against viral and non-viral pathogens* (adopted from Jones and Dangl, 2006). The ultimate amplitude of disease resistance or susceptibility is proportional to $[PTI - ETS + ETI]$. During phase 1, plants sense microbe-associated molecular patterns (MAMPs) and host danger-associated molecular patterns (DAMPs) via pattern-recognition receptors (PRRs) to induce PRR-triggered immunity (PTI). In phase 2, successful pathogens secrete effectors/suppressors into the plant interfering with PTI which results in effector-triggered susceptibility (ETS). In phase 3, plant recognizes directly or indirectly the effector molecule (indicated in red) by an NB-LRR protein which results in triggering effector-triggered immunity (ETI), a boost version of PTI passing a threshold for induction of hypersensitive response (HR) and programmed cell death (PCD). In phase 4, a new selection of pathogen isolates, that have lost the red effector and gained a new effector (indicated in purple), is done. This process helps pathogens to overcome ETI, however selection pressure favour new plants possessing new NB-LRR allele recognising one of the newly acquired effector, resulting again in ETS.

2. MICROBE-ASSOCIATED MOLECULAR PATTERNS TRIGGERED IMMUNITY (MTI)

Evolutionary conserved microbial signatures (also termed as exogenous elicitors) are counterparts of animal pathogen-associated molecular patterns (PAMPs). They occur in both, pathogenic and non-pathogenic microbes, and because the basal line of plant defence does not distinguish between mutualistic and parasitic symbiosis, the term Microbe-Associated Molecular Patterns (MAMPs) has been introduced (Schwessinger and Ronald, 2012). MAMPs are largely diverse in their chemical nature including oligosaccharides, (poly)-peptides, (glyco)-proteins and lipids; such as chitin, a structural component of the cell wall of fungi; flagellin, a protein subunit of bacterial flagella; lipopolysaccharides from outer membrane of gram-negative bacteria; xylanase, Pep-13, ergosterol, cold-shock proteins or oligogalacturonides (Boller and Felix, 2009; Henry et al., 2012; Nürnberger et al., 2004). Some of the MAMPs are only perceived by a narrow range

of plant species belonging to a single family (Felix and Boller, 2003; Ron and Avni, 2004). Such an example is the EF-Tu (elongation factor thermo unstable) recognised only in the *Brassicaceae* family (Kunze et al., 2004). On the other hand, other MAMPs such as chitin, lipopolysaccharides and flagellin, trigger defence responses in various host species, although there is difference to a certain extent in specificity and perception efficacy for a plant family or species (Zipfel et al., 2006). For all these reasons, MTI typically wards off multiple microbes, no matter whatever pathogenic or not, likely because of the conserve structure of MAMPs across diverse microorganisms. Recent studies suggested that endogenous danger-associated molecular patterns (DAMPs), released from the host plant by enzymatic or mechanical processes controlled by the pathogen, help further amplifying MTI to establish a robust systemic plant immune response (Reimer-Michalski and Conrath, 2016).

2.1 PERCPEPTION OF MAMPs

Today it seems that in plants the recognition of a wide range of proteinaceous, carbohydrate or lipophilic MAMPs or DAMPs rely only on plasma membrane localized receptor kinases (RKs) or receptor-like proteins (RLPs) (Böhm et al., 2014; Cui et al., 2015; Zipfel, 2014). This is in contrast to mammals in which both, surface localized (e.g. TLRs) and intracellular (e.g. NLRs) immune receptors, have been shown to recognise PAMPs (Maekawa et al., 2011). A typical receptor like-kinase consists of a variable extracellular leucine-rich repeat (LRR) ligand-binding domain, resembling that of Toll-like receptors in animals, a single transmembrane domain and an intracellular serine/threonine kinase-signalling domain and bear structural similarities to animal receptor-tyrosine kinases (RTKs)

(Böhm et al., 2014; Shiu and Bleecker, 2001). Although numerous MAMPs and their corresponding PRRs have been reported, only a few of them are well characterized. Some of the best characterized MAMP-PRR recognitions in bacteria perception are Flg22:FLS2 and EF-Tu:EFR. Flg22 (a 22-amino-acid epitope from the N-terminus of flagellin) binding to FLS2 (flagellin-sensitive 2) leads to the rapid forming of heterodimers with another LRR-RK BAK1/SERK, what is required for the full activation of FLS2 and flg22-triggered immune signalling (Figure 2) (Chinchilla et al., 2006; Zipfel et al., 2006). Interestingly, it is becoming increasingly apparent that BAK1, and related SERK proteins, associate with several additional LRR-RKs or LRR-RLPs like brassinolide (BL) receptor BR1 or elicitor receptor ELR (Figure 2) (Du et al., 2015; Zipfel, 2014).

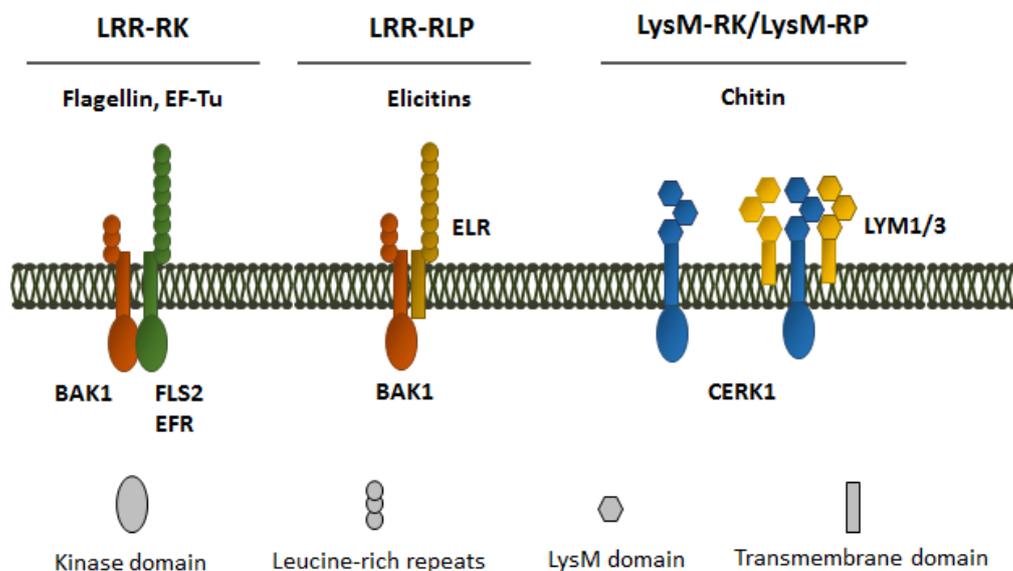


Figure 2. Various types of plasma membrane immune receptors for perception of MAMPs (adopted from Böhm et al. 2014). Leucine-Rich Repeat Receptor Kinase (LRR-RK) and Leucine-Rich Repeat Receptor-Like Proteins (LRR-RLP) type immune receptors sense proteinaceous patterns. The conserved LRR-RKs FLS2 and EFR recognise flagellin (via the fls2 epitope) and Elongation Factor Tu (EF-Tu), respectively and form heterodimeric complex with LRR-RK BAK1. The conserved LRR-RLP ELR form heterodimer with LRR-RK BAK1/SERK3 and recognise elicitors domain. Lys-M type immune receptors recognise GlcNAc-containing carbohydrate ligands by LRR-RK CERK1 or by LRR-RLP forming heteromeric complexes with LysM-RK CERK1.

In perception of fungi the recognition of chitin, a major constituent of fungal cell walls and classical MAMP, has been studied for decades in plants. Lysine motif receptor kinases, (LysM-RKs) with the extracellular LysM domain recognising *N-acetylglucosamine* (fungal chitin, bacterial peptidoglycan or bacterial nodulation factor) and intracellular Ser/Thr kinase domain, play a crucial role in perception of fungal chitin (Figure 2). However, different plants use distinct mechanisms for chitin perception; for example, chitin elicitor receptor kinase (CERK1) of *Arabidopsis* or chitin oligosaccharide elicitor-binding protein (CEBiP) in rice exhibiting three and two LysM extracellular domains, respectively (Figure 2) (Kaku et al., 2006; Miya et al., 2007).

In perception of oomycetes, recently a receptor-like protein ELR (elicitin response) responsible for extracellular recognition of elicitins, a molecular pattern conserved in *Phytophthora* species, was identified in the wild potato *Solanum microdontum*. Noticeably, similarly to flg22, ELR associates with the immune co-receptors BAK1/SERK (Chaparro-Garcia et al., 2011; Du et al., 2015).

2.2 PATHOGENESIS RELATED PROTEINS AND SYSTEMIC PLANT IMMUNITY

After the perception of MAMPs, in many plant species, a massive induction of inducible defence-related proteins has been described (Dadakova et al., 2015; Keller et al., 1996; Klemptner et al., 2014; van Loon et al., 2006). These proteins are called pathogenesis-related proteins (PRs) and due to the common features they have been classified 17 families (Table 1).

Table 1. Individual families of PR proteins (van Loon et al., 2006)

Family	Type member	Function
PR-1	Tobacco PR-1	unknown
PR-2	Tobacco PR-2	β -1,3-glucanase
PR-3	Tobacco P, Q	chitinase
PR-4	Tobacco `R'	Chitinase
PR-5	Tobacco S	thaumatin-like
PR-6	Tomato Inhibitor I	proteinase-inhibitor
PR-7	Tomato P	endoproteinase
PR-8	Cucumber chitinase	Chitinase
PR-9	Tobacco `lignin-forming peroxidase'	peroxidase
PR-10	Parsley `PR1`	ribonuclease-like
PR-11	Tobacco class V	chitinase
PR-12	Radish Rs-AFP3	Defensin
PR-13	<i>Arabidopsis</i> THI2.1	Thionin
PR-14	Barley LTP4	lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate-oxidase-like
PR-17	Tobacco PRp27	Unknown

The common features of these proteins are usually antimicrobial activities *in-vitro* through hydrolytic activities on cell walls, contact toxicity, and an involvement in defence signalling. Three base signalling compounds included in the process of PRs induction are salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). The production of PR proteins is related to the process of systemic plant immunity covering System Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) (van Loon et al., 2006). In SAR, which wards off diverse biotrophic pathogens, the presence of SA-induced PR-type proteins is likely to contribute to some extent to the enhanced defensive capacity. On the other hand, in ISR that is activated by root-colonizing grow/yield promoting bacteria and fungi, no defence-related proteins are present in induced leaves before challenge, but upon infection, the activation of JA- and/or ET-responsive genes in particular is accelerated and enhanced, in

a phenomenon called defence priming (Conrath et al., 2015). Consequently, the activation of both systemic immune responses, SAR and ISR, can result in an additive effect, however a negative cross-talk of the SA- and JA/ET signalling pathways has been reported as well (van Loon et al., 2006; Reimer-Michalski and Conrath, 2016).

3. ERGOSTEROL AS AN EXAMPLE OF AN ORPHAN FUNGAL MAMP

Ergosterol (5,7 – diene oxysterol), a principal compound of the fungal plasma membrane which has never been found in plants, is perceived by many plant species as an elicitor (Amborabé et al., 2003; Granada et al., 1995; Klemptner et al., 2014; Laquitaine et al., 2006; Rossard et al., 2010). Sterols are essential for the organisation and structure of eukaryotic cells plasma membranes when ergosterol is the major component in lower eukaryotes, whereas cholesterol and sitosterol are the most abundant sterols in higher eukaryotes and plants, respectively (Roche et al., 2008; Xu et al., 2001). These steroids differ structurally, with ergosterol having two additional double bonds (at the positions C7-C8 and C22-C23) and a methyl group at the C24 position of the side chain (Figure 3).

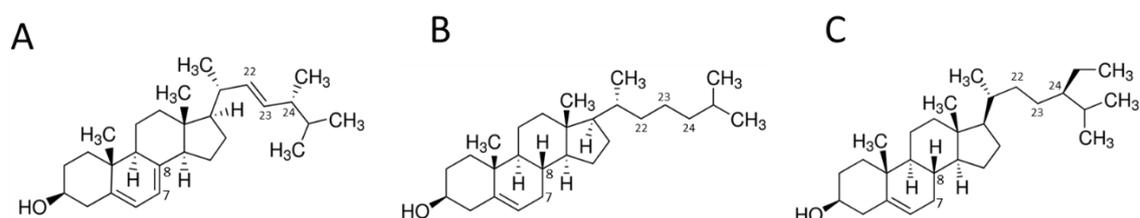


Figure 3. Chemical structure of ergosterol (A), cholesterol (B) and β -sitosterol (C) showing similarities and differences. Compared to cholesterol, ergosterol has unsaturated positions C7-C8 and an additional methyl group at the position C24 and sitosterol has an additional ethyl group at position C24.

These structural differences presumably allow the plant cell to recognize ergosterol as a typical MAMP, when it has been shown, that many aspects of the early response to ergosterol treatment are similar to that of chitosan, including desensitization phenomenon (Amborabé et al., 2003).

3.1 *ERGOSTEROL INDUCED DEFENCE REACTION IN PLANTS*

Previously, using various plant models, ergosterol treatment has been shown to induce an early phase of defence reaction characterized by changes in membrane potential, cytosolic calcium level and modification of H⁺ flux or production of reactive oxygen species (ROS), even in nano-molar concentration (Amborabé et al., 2003; Kasparovsky et al., 2004; Rossard et al., 2006; Vatsa et al., 2011). In the case of a plant infiltration, the ergosterol insolubility in water was increased by dissolving it in 2-hydroxypropyl- β -cyclodextrin, which is generally used for solubilisation of different lipophilic compounds (Lochman and Mikes, 2006; Uekama, 2004). Exogenous application of ergosterol to tobacco plants induced an accumulation of salicylic acid (SA) together with transcripts of phenylalanine-ammonia lyase (PAL), sesquiterpene cyclase (EAS) and acidic pathogenesis-related proteins PR1a, PR1b, PR3Q and PR5 (Figure 4) (Lochman and Mikes, 2006).

The PAL represents an important regulatory enzyme of secondary metabolism that has important roles in plants defence against pathogens (Dixon and Paiva, 1995; Howles et al., 1996). As the synthesis of salicylic acid is closely related to PAL activation, the measured

regulation of enzyme activity after an ergosterol application is not surprising (Dadakova et al., 2013).

EAS is an enzyme that plays an important role in the synthesis of sesquiterpene phytoalexins in tobacco in a response to various stress stimuli, pathogen and fungal elicitors. An increased concentration of capsidiol after an ergosterol treatment in cell suspension has been demonstrated (Kasparovsky et al., 2004). However, compared to previously used elicitors such as cryptogein or cellulase from *Trichoderma viridae*, which show a maximum expression after 8 hours following the treatment, the induction time for EAS in the case of ergosterol treatment shows a gradual increase within 24 hours (Lochman and Mikes, 2006), similar to that previously observed for methyljasmonate (Keller et al. 1998; Mandujano-Chávez et al., 2000).

The increasing expression of the acidic PR1a protein is usually used as a marker of systemic acquired resistance (SAR) although some recent studies demonstrated that its expression may not be linked with neither development of SAR nor with an effective resistance phenotype (Block et al., 2005; Conrath, 2006). For the group of PR3 proteins, results of previous studies have indicated a chitinase activity leading to decreasing susceptibility to infection by fungi with chitin cell walls (Sela-Buurlage et al., 1993). For the group of PR5 proteins called thaumatin-like proteins, an antifungal activity has been demonstrated (Vigers et al., 1992).

Noticeably, ergosterol treatment did not result in the accumulation of a transcript for the basic PR1 protein and the expression of proteinase inhibitors I and II were suppressed (Lochman and Mikes, 2006). Proteinase inhibitors (PIs) have been characterized in various plant species. An induction of their expression after a wounding, TMV infections

and application of jasmonic acid was demonstrated (Linthorst et al., 1993; Park et al., 2000). A central signalling molecule in this pathway leading to the accumulation of PIs and basic PR proteins is jasmonic acid produced in the octadecanoid pathway. Many studies assumed the existence of a cross-talk between at least two main signalling pathways: SA dependent and JA dependent pathways (Heil and Bostock, 2002). In both tobacco and tomato, SA inhibited the octadecanoid pathway and JA synthesis (Baldwin et al.; Niki et al., 1998; Pena-Cortés et al.). Moreover, the accumulation of PIs in tomato in a response to wounding was strongly reduced by the application of salicylic acid (Doares et al., 1995). Similarly, SA-mediated inhibition of the octadecanoid pathway downstream of JA has been described (Engelberth et al., 2000). However, an acceptable model for the cross-talk between these two pathways has not been found up until now.

3.2 SIGNALLING PATHWAY TRIGGERED BY ERGOSTEROL

Unfortunately, the mechanisms of ergosterol recognition by plant cell have not been described yet. It is hypothesised, that plants either possess an ergosterol receptor, or that ergosterol uptake results in perturbations of a lipid raft structure because of the ability of ergosterol to form very stable microdomains (Klemptner et al., 2014). Although the signalling transduction pathway after perception of ergosterol has not been fully elucidated to date, some general aspects were already described.

3.3 THE ROLE OF SA AND SPERMINE

The observed accumulation of the PAL transcript and an increase in lipoxygenase (LOX) and phospholipase A₂ (PLA₂) activity after ergosterol treatment imply a possible involvement of both, SA and JA signalling, in ergosterol perception (Dadakova et al., 2013;

Lochman and Mikes, 2006). In the case of PAL, an up-regulation of the PAL gene caused an increased PAL activity due to elevated protein levels. Furthermore, a very good correlation between this increase and accumulation of SA was shown (Lochman and Mikes, 2006). On the other hand, measured changes in the NaLOX3 transcript after ergosterol treatment corresponded well to changes in LOX activity in *Beta vulgaris* leaves measured previously, with a rapid and transient increase peaking in the first two hours and subsequent slight decrease below basal level around 3 h (Dadakova et al., 2013; Rossard et al., 2010). However, no JA accumulation after the ergosterol application was detected (Dadakova et al., 2013). This observation corresponds to the results with a potato homolog (LOX-H3) where its depletion does not result in a reduced accumulation of the wound-induced JA level suggesting that LOX-H3 may play a regulatory role over other activities (Royo et al., 1999) and the role of NaLOX3 in JA synthesis is contentious. Surprisingly, an elevated level of free polyamine spermine, able to trigger acidic PR proteins accumulation in plants, was measured after ergosterol application (Dadakova et al., 2013). These findings indicate that SA and spermine signalling pathways are more likely involved in ergosterol elicitation than JA signalling (Figure 4). This is further supported by the above mentioned observations that ergosterol treatment triggers an accumulation of transcripts only for the acidic form of PR1 protein and suppresses accumulation of transcripts for PI-I and -II induced by wounding (Lochman and Mikes, 2006).

3.4 THE ROLE OF NO AND CALCIUM

Among plants, NO plays a key role in diverse (patho) physiological processes, e.g. hormonal, wounding and defence responses, stomatal closure, and cell death regulation. In plants, NO is generated by NOS-like enzymes, nitrate reductase or non-enzymatic

processes (Wendehenne et al., 2004; Wilson et al., 2008). The response to NO may involve a signalling through the cGMP, cADPR and Ca²⁺ pathways or activation of a MAP kinase signalling pathway (Wendehenne et al., 2004; Wilson et al., 2008). The observed NO accumulation in ergosterol treated cells by DAF-FM fluorescence probe and suppression of ergosterol induced accumulation of PR1a, PR5 and tPOXC1 transcripts by the scavenger of NO cPTIO and inhibitor of NOS-like activity L-NMMA implies that NO participates in activating defence related transcripts after ergosterol treatment and its production is controlled by a NOS-like enzyme (Dadakova et al., 2013). However, the inhibitor of NOS-like enzymes L-NMMA did not modify ROS production after the application of ergosterol to a tobacco cell suspension suggesting that NO does not directly participate in the activation of NADPH oxidase activity which was measured previously in different plant species (Dadakova et al., 2013; Kasparovsky et al., 2004; Rossard et al., 2006, 2010).

It has been shown that ergosterol induces a concentration-dependent elevation of calcium in the cytosol. The ergosterol-triggered Ca²⁺ increase is biphasic, where the first phase corresponds to the influx from the extracellular space and the second phase corresponds to the release of calcium from internal stores via IP₃ and cADPR sensitive channels (Figure 4) (Vatsa et al., 2011). Moreover, it has been shown that the cADPR channels are involved in both, NO- and SA-mediated pathways, leading to the activation of the PR1a gene (Klessig et al., 2000). Ruthenium red, a known inhibitor of cyclic ADP-ribose dependent Ca²⁺ channels, only inhibited the ergosterol-induced accumulation of PR1a transcript (Dadakova et al., 2013). Durner et al. showed that addition of cADPR causes increased the accumulation of mRNAs encoding PAL and PR1a protein in tobacco. The same pathway is likely to be important in ergosterol-triggered PR1a transcript induction (Figure 4). On the other hand, low or no inhibition of ergosterol-induced accumulation of tPOXC1

and PR5 transcripts indicates the involvement of other regulation mechanisms that are not fully dependent on Ca^{2+} channels located on the membranes of internal stores (Dadakova et al., 2013). Moreover, almost the same pattern of inhibition for the ergosterol-induced accumulation of PR1a, PR5 and tPOXC1 transcripts was observed with the inhibitor of calmodulin/ Ca^{2+} dependent steps W7 (Dadakova et al., 2013).

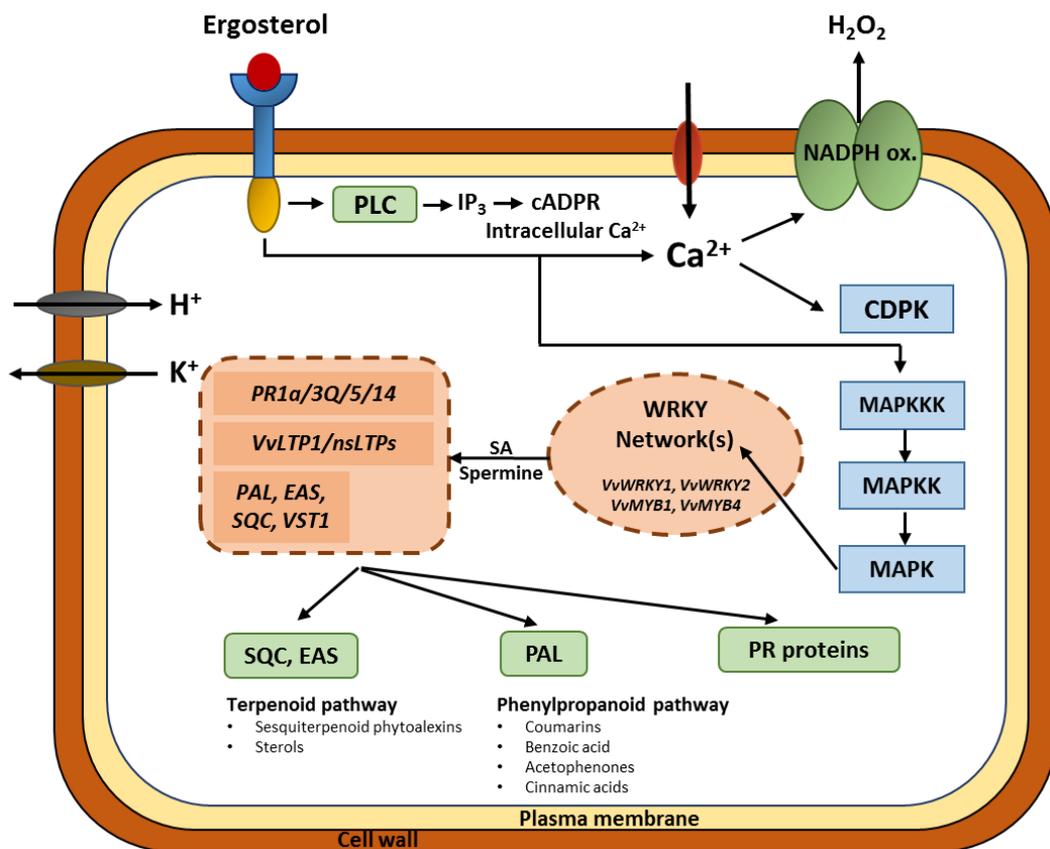


Figure 4. Summary of known /putative mechanisms taking a role in ergosterol perception (adopted from Klemptner et al. 2014). The scheme illustrates the perception of ergosterol by a hypothesised receptor, based on the assumption that ergosterol may be perceived in the same manner like other MAMPs. Ergosterol perception has been shown to trigger a Ca^{2+} influx as well as Ca^{2+} release from intracellular stores. The already known pathways that are involved in the defence response following ergosterol treatment are shown including putative WRKY transcription factors in the nucleus activated through various upstream interactions by MAPK-based signalling. Sesquiterpene cyclase (SQC), 5-epi-aristolochene synthase (EAS) and phenylalanine-ammonia lyase (PAL) represent important metabolic enzymes contributing to the production of specific ergosterol-

induced metabolites. The genes, proteins and metabolites included in this scheme have resulted from studies in tobacco and grapevine.

Recently, the role of both calcium-dependent and -independent pathways in ergosterol elicitation has been shown (Vatsa et al., 2011). Whereas the activation of NADPH oxidase and inhibition of H⁺-ATPase were clearly calcium-dependent, the activation of two mitogen-activated protein kinases (SIPK and WIPK) during the first minutes after ergosterol treatment was shown to be calcium independent (Figure 4). With respect to the elevated spermine content triggered by ergosterol (Dadakova et al., 2013) a good candidate for the observed calcium independent pathway is, the spermine pathway, which role in the induction of both transcripts, *PR5* and *tPOXC1* has previously been demonstrated (Hiraga et al., 2000; Yamakawa et al., 1998).

3.5 CONTRIBUTION TO GIVEN PROBLEMATIC

Fungal pathogens are a significant threat to crop production. Ergosterol, a principal compound of the fungal plasma membrane, which has never been found in plants, is one of the classical MAMP molecule together with chitin, chitosan or β -glucans. Therefore, understanding the mechanism of its participating in the perception by plants is one of the challenge in discovering of plant's innate immunity.

Within our work, we firstly demonstrate the ability of ergosterol to trigger the expression of some defence related genes in plants. We found a specific induction of acidic forms of PR proteins families and enzymes participating in the defence response, such as phenylalanine ammonia lyase and sesquiterpene cyclase. Further, a possible cross-talk between the signalling pathways of salicylate and jasmonate was observed. Moreover, we studied in detail the signalling pathway following the ergosterol perception in plants. To

understand the sequence of the signalling cascade, several representative steps involved in the synthesis of crucial signalling molecules were targeted using specific inhibitors. The results showed an important role of calmodulin-dependent protein kinases and nitric oxide in SA-dependent pathway, resulting in the expression of defence-related genes. However, at the same time the presence of SA-independent spermine pathway was demonstrated. Our results from these studies significantly contributed to understanding the mechanism following ergosterol perception in plants and have been cited several times in prestigious journals.

4. ELICITINS AS AN EXAMPLE OF A TYPICAL OOMYCETE MAMP

Oomycetes are a distinct class of fungus-like eukaryotic microbes, many economically significant diseases of crop species as the potato late blight, caused by the pathogen *Phytophthora infestans*. On the other hand, oomycetes also include the mycoparasite *Pythium oligandrum*, which is used as a biocontrol agent against pathogenic fungi. Thus understanding the mechanisms taking place in the oomycal infection process could lead to new methods providing a durable resistance. Oomycete plant pathogens exhibit a biotrophic, necrotrophic or hemibiotrophic lifestyle. Among the biotrophic oomycetes, completely reliant on the host tissue are *Hyaloperonospora parasitica* or *Plasmopara viticola* causing the white rust. Typical representatives of hemibiotrophic oomycetes are *Phytophthora* species, some of which have a potential to infect a wide range of different plant species. Necrotrophs are represented e.g. *Pythium ultimum* (Fawke et al., 2015). During the evolution, some parasitic *Phytophthora* and *Pythium* spp. have lost the ability to synthesize their own sterols and must pick-up sterols from the host cell

membranes. From culture filtrates of non-pathogenic *Phytophthora* spp. on tobacco, *P. cryptogea* and *P. capsici*, small proteinaceous elicitors named elicitins (namely cryptogein and capsicein) were isolated (Ponchet et al., 1999). Elicitins are members of a family of conserved lipid transfer proteins with sterol-binding and elicitor activity, catalysing an in-vitro sterol transfer between liposomes and micelles (Mikes et al., 1997, 1998). However, there is still missing a clear *in-vivo* evidence of their involvement in sterol uptake because a *P. infestans* strain lacking elicitin INF1 remains pathogenic (Kamoun et al., 1997, 1998). Today, elicitins are among the most well-known oomycetes MAMPs which have been shown to induce the hypersensitive response (HR) in several plants, such as *Nicotiana* species and some radish and rape cultivars (Kamoun et al., 1998; Panabieres et al., 1995; Ponchet et al., 1999; Ricci et al., 1989). Elicitins represent a highly conserved family of proteins characterised by the “elicitins signature” distinguished by their size (98 aminoacids), lack of amino acid tryptophan, histidine and arginine and abundance of a few amino acids, such as serine, threonine (30 % of the protein), and leucine (about 10%), and the presence of six cysteine residues located in conserved positions responsible for three structurally determinant disulphide bridges. Based on the primary structure of elicitins, three different classes have been identified. Elicitins from class I contain only the elicitin domain of 98 amino acids and can be further separated according to their respective *pI* to class I α including acidic α -elicitins ($pI < 5$) and class I β including basic β -elicitins ($pI > 7.5$). Despite the presence of both within the genome of respective *Phytophthora* spp. subclasses are usually present, compared to α -elicitins, β -elicitins were found to be secreted by a restricted range of species and appear to be ancestors of other elicitins (Ponchet et al., 1999). Class II contains hyperacidic elicitins with the sequence length of about 103 amino acids and net charges ranging from -6 to -10 which transcripts have not been

observed within the corresponding species up today. The last class III contains elicitin-encoding sequences from *P. infestans* having app. 70 amino acids long C-terminal sequence rich in Ala, Ser and Thr residues, representing a possible O-glycosylation domain.

The three-dimensional structure of elicitins were firstly determined for a basic elicitin cryptogein secreted by *Phytophthora cryptogea*. The three-dimensional structure of cryptogein is composed of five α -helices, one β -sheet, and one ω -loop arranged in a unique protein fold (Figure 5) (Boissy et al., 1996; Gooley et al., 1998). The ω -loop present in the structure of cryptogein is very flexible and highly conserved, which suggests it has an important function. The study of conformational changes, due to pH and cryptogein concentration, demonstrated the ability of cryptogein to dimmerize (Gooley et al., 1998) which was later proved by the published X-ray structure of the elicitin cinnamomin, secreted by *Phytophthora cinnamomi* crystallizing as a homodimer (Figure 5) (Rodrigues et al., 2006).

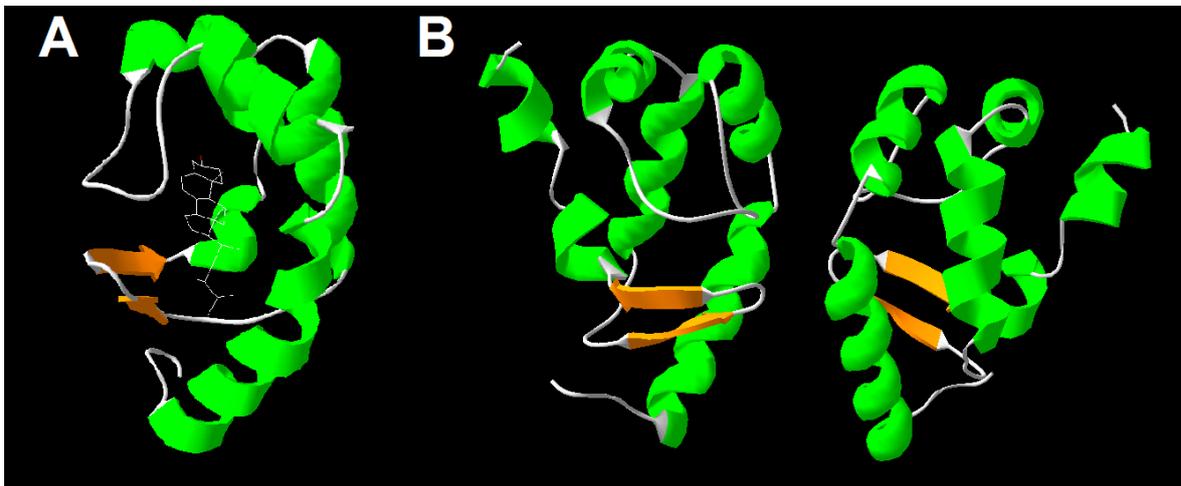


Figure 5. Structure of elicitin β -cryptogein (A) and cinnamomin (B). Cryptogein structure composed of five α -helices, one β -sheet, and one ω -loop is shown with a molecule of ergosterol located in the hydrophobic cavity (Boissy et al., 1999). β -Cinnamomin crystalized as a homodimer with opposite orientation of monomer subunits (Rodrigues et al., 2006). Both structures were determined by X-ray crystallography.

Most of the further characterisation of interactions between the elicitors and plants has been carried out on tobacco plants, by using cryptogein representing a very efficient β -elicitor of class I secreted by *Phytophthora cryptogea*. In tobacco cells the perception step is followed either activation of protein kinases (PK) or inhibition of protein phosphatases (PP), triggering the Ca^{2+} influx, which, in turn, triggers ROS production, MAPK activation, anion effluxes and plasma-membrane depolarization, and glucose (Glc) import inhibition, or may lead to H^+ -ATPase inhibition (for a review, see Garcia-Brugger et al. 2006).

4.1 CHARACTERIZATION OF ELICITINS BINDING SITE

Consequent cryptogein binding studies and crosslinking experiments performed on tobacco plasma membrane resulted in the characterization of a single, low-abundance class of binding site with a K_d value of 2 nM, characterized as a glycosylated heterodimeric protein (Bourque et al., 1999; Wendehenne et al., 1995). Further, Svozilova et al. studied the interaction of cryptogein with high-affinity binding site in real-time using a piezoelectric biosensor and found values of kinetic rate association $k_a = 5.74 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and kinetic rate dissociation $k_d = 6.87 \cdot 10^{-4} \text{ s}^{-1}$ constants, respectively. The calculated kinetic equilibrium dissociation constant ($K_D = 12.0 \text{ nM}$) was six times lower than that calculated in previous a study but the obtained value of k_a was substantially higher than the previous corresponding value from the literature ($k_a = 1.35 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$) (Bourque et al., 1999; Svozilová et al., 2009). This discrepancy should be mainly because of using another approach compared to previous studies (Bourque et al., 1999; Wendehenne et al., 1995) using radioactive labelling and not real-time measurement. Moreover, in the study by Svozilova et al., the exact concentration of binding sites was carefully determined, providing only the number of

binding sites accessible on the outer surface of membrane vesicles (Svozilová et al., 2009). Recently, in the wild potato *Solanum microdontum*, a receptor-like protein ELR (elicitin response) mediating the extracellular recognition of the elicitin domain was demonstrated, although the binding to elicitins still needs to be proved (Du et al., 2015). ELR protein associated with a well-known central regulator of cell surface-mediated immunity BAK1 (BRI1 associated kinase 1), also known as SERK3 in solanaceous plants, undergoes complex formation with PRRs after ligand binding (Du et al., 2015). Previously, such a response to elicitin INF1 from *P. infestans* was just dependent on BAK1/SERK3 in tobacco (Chaparro-Garcia et al., 2011).

Although α - and β -elicitins have been shown to bind with an equivalent affinity to the same high affinity binding site on the plasma membrane (Bourque et al., 1998), one important difference was determined; after an application on decapitated tobacco plants, β -elicitins were shown to be 50- to 100-fold more active in inducing a distal HR and systemic resistance than α -isoforms. Moreover, Bourque et al. showed that β -elicitins in a tobacco cell suspension are at least 10-times more efficient in inducing extracellular pH changes, reactive oxygen species (ROS) production or Ca^{2+} influx compared to α -elicitins (Bourque et al., 1998). Even though the elicitin binding seems to be a prerequisite for the induction of the plant defense response, e.g. the AVR9/Cf-9 interaction in tomato or NIP1/Rrs1 in barley, an effective response is only observed in the presence of a third interacting component (Bourque et al., 1999; van't Slot et al., 2007; Wulff et al., 2009). Kanzaki et al. showed that the elicitin INF1 could interact with the intracellular kinase domain of NbLRK1 kinase (Kanzaki et al., 2008). Although at first glance their results suggesting the intracellular recognition of elicitins seem to be enigmatic, they fully correspond with the measured stimulation of clathrin-mediated endocytosis by the elicitin cryptogein in

tobacco cells or localization of the elicitor quercetin inside cells of host oak plants by immunocytology (Brummer et al., 2002; Leborgne-Castel et al., 2008). Finally, a ligand-induced receptor endocytosis has been suggested to be involved in the activation of plant defense mechanisms (Robatzek, 2007).

In the next chapters, three main factors with a suggested role in different biological activity of α - and β -elicitors are discussed.

4.2 THE ROLE OF STEROL TRAPPING IN ELICITINS BIOLOGICAL ACTIVITY

Naturally occurring elicitors show a similar binding characteristics represented by a 1:1 sterol:protein stoichiometry and the dissociation constants in the same range. Despite these similarity, elicitors exhibit differences in the kinetics of sterol trapping and exchange between liposomes or micelles (Mikes et al., 1998; Vauthrin et al., 1999). The most efficient elicitor is the β -elicitor cryptogin (from *P. cryptogea*), the less efficient being α -elicitors parasiticein and capsicein, secreted by *P. parasitica* and *P. capsici*, respectively. Together with differences in triggering defence cell responses between α - and β -elicitors, the sterol-binding hypothesis was proposed (Figure 6).

Osman et al. (2001) studied the link between elicitor and sterol-loading properties with the use of site-directed mutagenesis of the 47 and 87 cryptogin tyrosine residues, playing an important role in sterol binding based on crystal structure of an engineered cryptogin-ergosterol complex (Boissy et al., 1999). The results demonstrated a link between elicitor and sterol-carrier activity of elicitors when the authors suggested that the sterol binding induced conformational changes in the ω -loop which “activate” elicitors and allow them to bind effectively to high-affinity binding sites and trigger cell responses.

However, at the least, the affinities and activities of the Tyr47Gly mutant from this study need to be interpreted with caution. According to the proposed working scheme of elicitin action, an effect similar to that of sterol binding and hence similar protein activities should be expected for Tyr47Phe and Tyr47Gly mutants. Nevertheless, high variances in individual parameters between the Tyr47Phe and Tyr47Gly mutants were observed which could be explained by a structural change in the helix C evoked by glycine, the amino acid with the lowest tendency to form a helical structure (Pace and Scholtz, 1998) and unable to stack with the side chains of Pro42 and Tyr33, in contrast to phenylalanine.

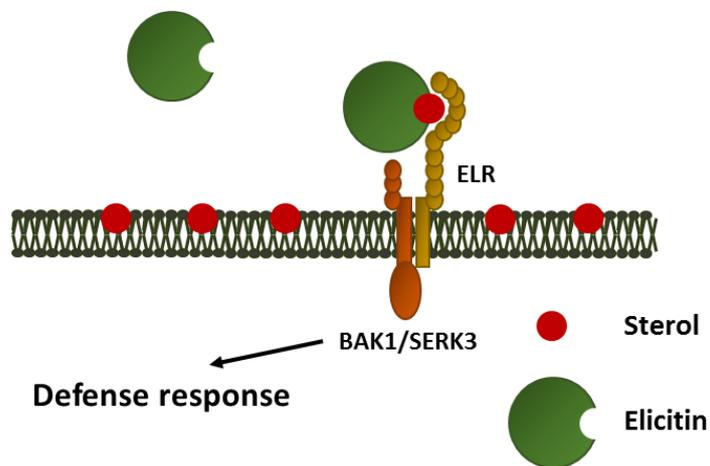


Figure 6. *Sterol-binding hypothesis in activity of elicitins.* Elicitin traps sterols from plant plasma membrane (PM) and such an “activated elicitin” is able to bind to high affinity binding site (ELR protein) on PM and activate plant defence response.

Thus, to confirm these results, Lochman et al. carried out a site-directed mutagenesis on cryptogein to introduce mutations Met35Phe/Met59Trp, Met35Phe/Met59Trp /Ile63Phe, Met35Trp/Met59Trp, Leu19Arg and Leu15Trp/Leu36Phe located mainly in the hydrophobic cavity (Lochman et al. 2005). All mutants showed altered abilities to bind sterols and fatty acids, nevertheless based on the results authors suggested, that the conformational change of the ω -loop may be necessary to trigger early

defence responses, such as the synthesis of reactive oxygen species (ROS), but not for the activation of later responses such as PR-protein expression and cell necrosis . However, since the mutations considered in that study were targeted to the ω -loop region they not only affected the sterol- and phospholipid-binding properties of cryptogein, but also slightly modified both the ω -loop structure and the overall protein structure.

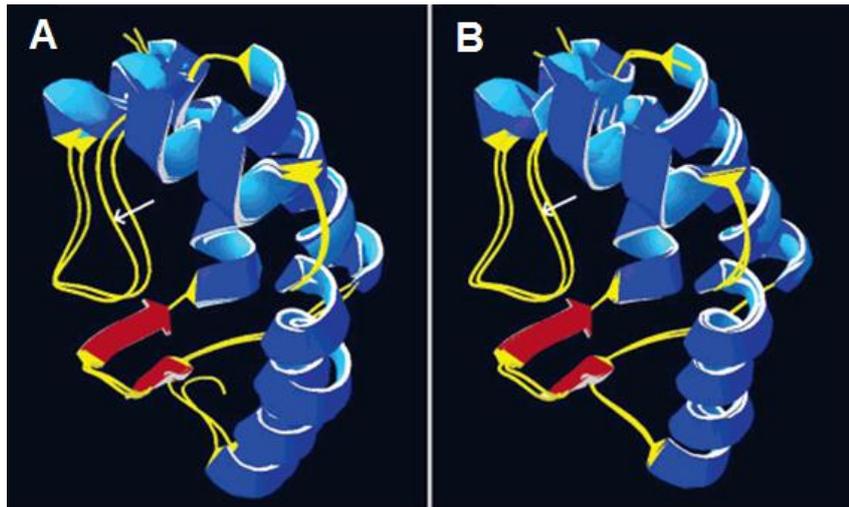


Figure 7. Superposition of the free wild type, dehydroergosterol bound wild type, and mutant structures of cryptogein. (A) Binding of dehydroergosterol to the cavity of cryptogein wild type induces a conformational change in the ω loop (arrow). (B) Superposition of the structures of wild type and the M35W/M59W mutant (arrow) of cryptogein. Changes induced by the mutation are similar to those induced by sterol binding (adopted from Lochman et al. 2005).

Even though, conclusions from these studies indicated some role of sterol binding in elicins biological activity, the conclusions drawn they were not fully concordant and apparent discrepancies remained to be explained. For this reason, the main goal of Dokládál et al. 2013 was to finally support or contradict the role of the elicitin-sterol complex in the induction of defence responses triggered by elicins. Based on previous results (Dobes et al., 2004), site-directed mutagenesis of the elicitin cryptogein was performed in order to modify the residues that have been proposed to be responsible for

sterol- and/or phospholipid-binding without modifying the ω -loop or overall protein structure. By this strategy three new cryptogein mutants were prepared; Leu41Phe mutant with a reduced ability to transfer fatty acids, Val84Phe mutant having a reduced ability to transfer sterols, and the double mutant Leu41Phe/Val84Phe having reduced ability to transfer sterols and fatty acids. Contrary to previous studies (Lochman et al., 2005; Osman et al., 2001), the Val84Phe mutant, with a substantially lowered ability to bind and transfer sterols, was as efficient as wt cryptogein in stimulating ROS production and induction of qualitative changes of intercellular proteome in proteins playing an important role in resistance processes. Surprisingly, the Leu41Phe mutant, with only a slightly lowered ability to bind and transfer sterols and Leu41Phe/Val84Phe double mutant showing almost no ability to transfer and bind sterols, were far less efficient in inducing the synthesis of ROS and proteome changes (Dokládál et al., 2012).

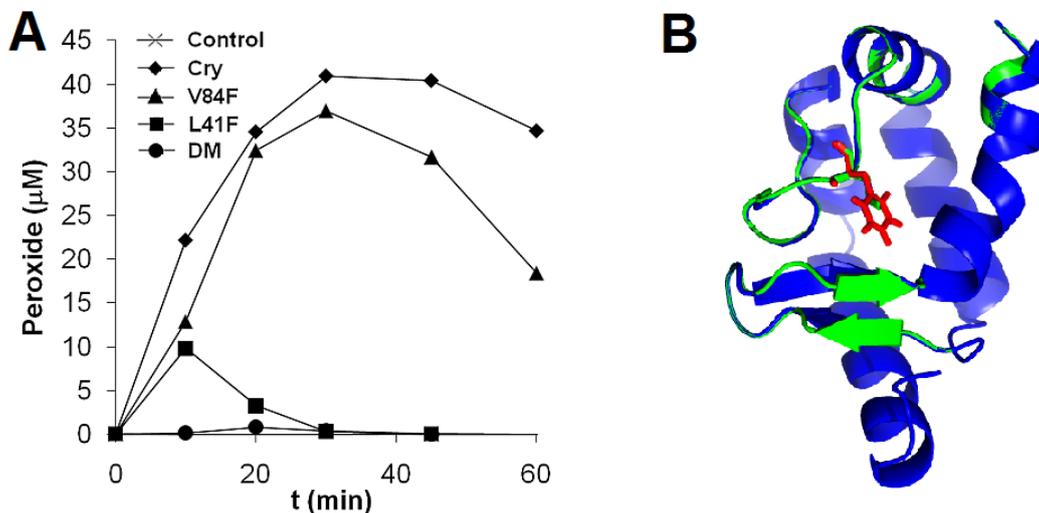


Figure 8. Extent of ROS production and structural alignment of the wild-type cryptogein (green) and the L41F mutant (blue) (A) ROS synthesis in tobacco cells in suspension stimulated by 10 nM elicitors - wt cryptogein (diamonds), V84F (filled triangles), L41F (squares), L41F/V84F (circles) and control (X) (B) The side-chains of the mutated residues (red) adopt their conformation without a disruption of the omega loop or entire protein structure (adopted from Dokládál et al. 2012).

This unexpected result was explained by the construction of a double mutant Leu80Phe/Val84Phe and detailed characterization of mutants' interaction with high-affinity binding on the plasma membrane, when mutation of a small leucine residue 41 for a large hydrophobic phenylalanine residue altered the interaction of the protein with this high-affinity binding site (Dokládál et al., 2012).

All these findings contradict previous study of Osman et al. (2001) suggesting the necessity of a change in the ω -loop for elicitors activation. At a first sight, the inconsistency of the obtained data with those published by Lochman et al. (2005), which suggested the lack of any relation between the ability of elicitors to induce the production of PR proteins together with tissue necrosis and sterol-binding properties, is clear as well. But if we take into consideration the fact that the residues Met35 and Leu36 in the ω -loop are highly conserved among elicitors, as in the case of the Leu41Phe mutant, their mutation to large bulky residues (tryptophan and phenylalanine) could have resulted in an altered interaction with the high-affinity binding site, which was not determined. Consequently, this effect could be responsible for a longer lag phase (especially at the lower concentration) before the pH changes and ROS production, but it had a minimal effect in the late phase of cell necrosis or expression of defence genes.

A final summary of all obtained results imply that the conformational change in the ω -loop induced by sterol binding might not be crucial factor in determining an elicitors' activity. So, it is obvious that the activity of elicitors is more dependent on the presence of specific residues than on the ω -loop structure; the most probable candidates being lysine residues in helices A and D of basic elicitors (Dokládál et al., 2012, Ptáčková et al., 2015). This assumption is further supported by the observed correlation between the necrotic

index and PI (Pernollet et al. 1993) and by a clear impact of the Lys13Val mutation in helix A on induction of a defence response in tobacco plants (Plešková et al., 2011).

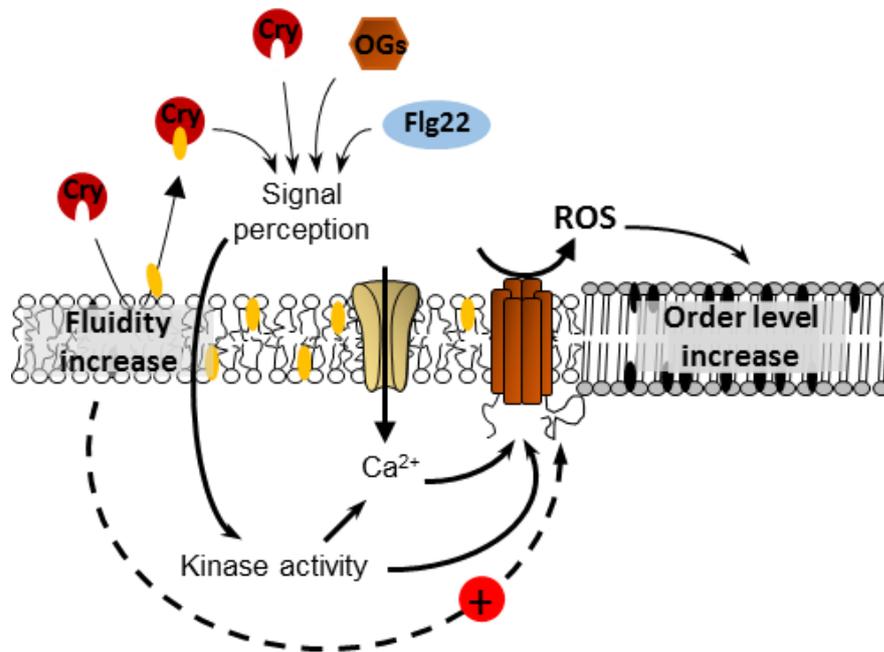


Figure 9. *Enhancing of elicitor-induced ROS production in tobacco cells.* Perception of MAMPs represented by cryptogein (Cry), flagellin (Flg22), and oligogalacturonides (OGs) triggers a kinase activation leading to calcium influx and activation of the NADPH oxidase-driven ROS burst, which in turn increases membrane order. In parallel, the sterol-trapping ability of cryptogein leads to an increase in membrane fluidity which enhances the ROS burst intensity compare to Flg22 or OGs (adopted from Sandor et al. 2016).

However, recently it was proved that the sterol-trapping activity of elicitors influence PM fluidity and might act as an enhancing factor of elicitor-induced ROS production in agreement with the synergistic effect obtained with a combination of cyclodextrin and the potent elicitor methyl jasmonate (Lijavetzky et al., 2008). On the basis of this results, a model assuming two roles for cryptogein was proposed. The first role is in triggering of a signalling cascade including the modification of membrane order independently on sterol-trapping activity and the second role is as a ROS production

enhancer through a sterol trapping from the PM, and thus increasing PM fluidity (Figure 9). This model of co-operative effect could explain how cryptogein would exhibit a strong ability to induce defence responses including cell death in tobacco cells (Hirasawa et al., 2004; Kadota et al., 2004, Ptáčková et al., 2015), whereas other classical MAMPs as flg22 and OGs trigger a signalling cascade without inducing cell death (Figure 9).

4.3 THE ROLE OF ELICITINS NET CHARGE IN THEIR BIOLOGICAL ACTIVITY

A rapid comparison of α - and β -elicitins provides some evident characteristics for each form, significantly differing in the induction of a distal hypersensitive response and resistance induction in plants. The most pronounced difference appears in the net charge which is the consequence of a different number of lysine residues. While the number of negatively charged Asp and Glu residues is among the basic and acidic elicitors almost similar, the presence of 6 Lys residues gives a positive charge to β -elicitors and the presence of only 2-4 Lys residues gives a negative charge to α -elicitors (Figure 10). The importance of elicitors net charge has been from the beginning by the observed correlation between the necrotic index and pI (Pernollet et al., 1993).

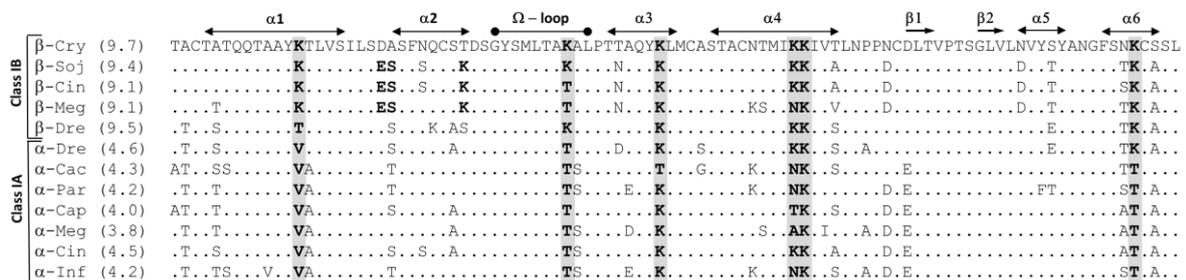


Figure 10. A comparison of selected α - and β -elicitors. The multiple sequence alignment of selected protein sequences of elicitors and cryptogein was performed using the CLUSTALW algorithm; the corresponding lysine residues are highlighted in bold.

Other structural differences were found among the amino acid sequences in the A helix of elicitins. The β -elicitins residues 13 and 14 are always polar amino acids (Lys and Thr) constituting for an interruption in the hydrophobic region, compared with α -elicitins (Figure 10). The importance of the residue Lys 13 in elicitins structure for the sterol trapping activity and induction of a defence response in tobacco plants was repeatedly shown (Hirasawa et al., 2004; O'Donohue et al., 1995; Plešková et al., 2011). Previously it was demonstrated that a cryptogein mutant Lys13Val is able to bind both sterols and fatty acids (Plešková et al., 2011) which correspond with the fact that the K_d values for sterol binding by acidic elicitins (containing valine at the amino acid position 13) are similar to those of the basic elicitins (Mikes et al., 1998). However, the sterol trapping capacity of Lys13Val mutant and acidic elicitins (e.g. cactorein or parasiticein) was about 5-fold lower, compared with the β -elicitin cryptogein (Plešková et al., 2011; Vauthrin et al., 1999). Based on these results it was proposed that the Lys13Val mutation eliminates the positive charge on the surface of cryptogein, which could be important for a correct protein positioning in the interaction between the protein and plasma membrane (Figure 11) (Plešková et al., 2011).

It is well known, that the electrostatic interactions between charged residues located on a protein surface are known to be important for a long-range recognition in biomolecular systems (Klein-Seetharaman, 2005). In an agreement with the previously proposed sterol-binding hypothesis the Lys13Val induce only a slight resistance against *P. parasitica* compared to cryptogein which was fully comparable with those induced by the acidic elicitin capsicein having at the amino position 13 valine. Moreover, this result was consistent with a significantly lower accumulation of chitinase (PR3Q) or thaumatin like protein (PR5) transcripts, for which an important role was described in the defence reaction against fungi (Plešková et al., 2011).

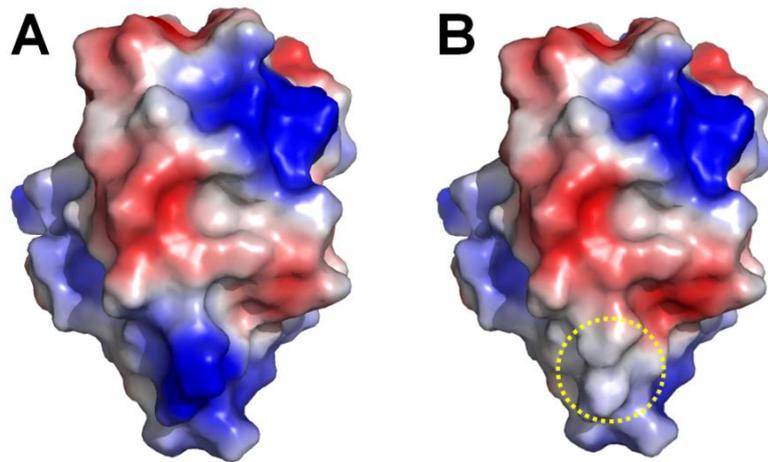


Figure 11. *Electrostatic potential surface maps of cryptogein and the Lys13Val mutant (adopted from Plešková et al. 2013). The Lys13Val mutation significantly alters the distribution of charges on the surface of cryptogein (A) and the Lys13Val mutant (B). The position of the mutated residue is indicated by a yellow circle.*

Even though, this result could be interpreted as a supporting argument for the sterol-binding hypothesis of elicitors biological activity, it must be interpreted with caution. Recently, Uhlíková et al. 2016 investigated the role of individual Lys residues in the ability to induce distal systemic resistance on a model of basic elicitor β -cryptogein when using site-directed mutagenesis of five Lys residues (Lys39, Lys48, Lys61, Lys62 and Lys94) were systematically replaced by Thr residues. In this study, basic biochemical properties of the proteins together with the induction of resistance to the pathogen *P. parasitica* were studied on tobacco plants. Similarly to the study by Dokladal et al. 2012, the obtained results did not support a crucial role of sterol binding for elicitors resistance induction when no correlation of the sterol-trapping activity of the mutants with their resistance induction activity was proved (Uhlíková et al., 2016). A mild decrease in the sterol transfer activity measured in a majority of the mutants was probably the result of changes in the protein surface charge, as discussed previously (Plešková et al., 2011).

In addition to the important role of lysine residues for elicitors sterol trapping activity, their role in the previously shown homo-dimer formation was suggested (Ponchet et al., 1999; Rodrigues et al., 2006). Particularly, the role of the residues 13 and 14, located in the helix A responsible for dimer formation, seems to be appealing. Uhlíková et al. 2016 proved by quartz crystal microbalance (QCM) experiments cryptogein homodimer formation in solution with a K_D value of 2.21 μ M, which was much closer to physiological conditions than calculated previously in NMR-based study (Gooley et al., 1998). However, the detailed QCM measurement of the kinetic parameters of cryptogein dimer formation resembled those of capsicein. Thus the homodimer formation does not seem to be a key process explaining differences in the biological activity of α - and β -elicitors. On the other hand, a chemical crosslinking of cryptogein dimer resulted in its largely reduced ability to induce necrosis and resistance in plants. To explicate these incongruous results of the homodimer role in biological activity of elicitors, a possible interaction of elicitors with another partner in plant, non-specific Lipid Transfer Protein 1 (nsLTP1), was studied (Uhlíková et al., 2016). The nsLTP1 protein was selected as a promising candidate because it behaves as an elicitor antagonist with known superimposition of helix 3 with helix A of cryptogein (Blein et al., 2002), its cell wall localization is assumed (Carvalho and Gomes, 2007) and an important role of LTPs in the key processes of plant physiology and plant defence signalling was proved (Champigny et al., 2013). Noticeably, there a strong correlation between the nsLTP1-elicitor complex formation kinetic and biological activity of individual used proteins was measured, including cryptogein variants carrying mutation in lysine residues (Lys39, Lys, 48 and Lys 94) and capsicein.

4.4 THE ROLE OF ELICITINS CHARGE IN THEIR MOVEMENT ACROSS THE PLANT

Most of the studies on elicitins have been carried out on tobacco plants with three different methods of their application: on the stem of decapitated plants, on the petiole of detached leaves or via a direct infiltration into the leaf mesophyll. The first two modes of treatment are used for measurement of induced distal resistance because it leads to the systemic movement of α - and β -elicitins across the plants or leaves (Devergne et al., 1992; Dorey et al., 1997; Keller et al., 1996; Uhlíková et al., 2016). Based on this treatment, β -elicitins were shown to be 50- to 100-fold more active in inducing of HR and systemic resistance against pathogens, compared to α - elicitins. In the latter mode, most of the α - and β -elicitins have approximately the same activity in inducing HR however they are restricted to the infiltration site without any movement to surrounding areas, inducing only local acquired resistance (LAR) against the pathogens (Dorey et al., 1997). Thus, it is clear that not the movement of elicitins in the phloem but the consequent transport from the phloem to companion and parenchyma cells plays a crucial role in their ability to induce systemic resistance in plants. This fact could be supported by previous findings that protein's properties play an important role in phloem loading/unloading (Niu et al. 2011). At the beginning, elicitin pl was considered as an important factor in this process because the elicitin penetration through the negatively charged cell wall could contribute to delay response of suspension cells to acidic elicitins despite their similar binding parameters to a high affinity binding site on the plasma membrane (Bourque et al., 1998). Nevertheless, no clear relationship between the charge of cryptogeins carrying mutation in Lys residues and their activity to induce resistance and HR disprove this hypothesis (Uhlíková et al., 2016). On the other hand, the noticeable role of Lys residues (Lys13, Lys39) and homodimer covalent crosslinking in elicitins ability to induce systemic resistance suggest an important

role of some partner in plant, forming a heterodimer complex with elicitin, facilitating the process of phloem unloading (Uhlíková et al., 2016). From this point of view, the described interaction of the studied proteins with nsLTP1, with a clear involvement of specific lysine residues, seems to be very promising for further studies.

4.5 CONTRIBUTION TO GIVEN PROBLEMATIC

Oomycetes, including *Phytophthora infestans* causing potato late blight, represent one of the most emerging pathogens on food crop. Elicitins are structurally conserved proteins secreted by *Phytophthora* and *Pythium* pathogenic species and they are recognised as oomycetes MAMPs.

The results of our studies significantly improved our knowledge about the role of the sterol-binding activity of elicitins in their ability to induce resistance in plants. By several studies using mutants affected in sterol binding we disproved previous results suggesting an essential role of sterol binding in elicitins biological activity, especially in their binding to potential high affinity binding site on plasma membrane. However, we demonstrate that the sterol-trapping activity could serve as an enhancer of ROS production through an increase in the plasma membrane fluidity. On the other hand, by using elicitins mutants affected in their surface charge, we demonstrated an important role of the lysine residues 13 and 39 in the ability of elicitins to induce systemic resistance in plants when we suggested an important role of some partner in plants, forming a heterodimer complex with elicitins and thus facilitating the process of phloem unloading. Within the framework of this topic, several long-term stays of our students in the cooperating laboratories of INRA in Dijon and Sophia-Antipolis have been made.

5. CONCLUSION

Today, in the period of an intensive agriculture practise, the cultivation of highly fertilized crops in large monocultures is widely used. Unfortunately, these types of crops are very sensitive to a wide spectrum of diseases and their cultivation requires the use of extensive amounts of pesticides and fungicides. In the past decades, the via transfer of resistance (*R*) genes was the main strategy to improve plant resistance. However, the durability of this resistance has been shown to be very limited because of the process of a rapid pathogen effectors evolution, and due to the species or strain-related specificity of effectors which fails against a broad spectrum of the attacking pathogens.

On the other hand, PTI, providing a broad-spectrum immunity, seems to be a very promising tool for engineering plants with an enhanced immunity. It is mainly due to the fact that MAMPs, which are specific and conserved in microbes, are very often crucial for their survival (e.g. flagellin for bacterial motility or ergosterol for fungal plasma membrane structure). Noticeably, a generally enhanced resistance after the interfamily transfer of genes encoding PRRs or other regulators of PTI was observed even though in some cases undesirable side effects in growth and development were found.

Further investigations of MAMPs perception mechanisms by plant cells emerge using novel large scale quantifiable tools (e.g. transcriptomics, proteomics or metabolomics). This will certainly provide comprehensive insights into the understanding of MAMPs interactions with plants. The long-time durable resistance should be achieved only through a combination of multiple PTI- and ETI-related genes together with deploying of a proper field management.

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7. PUBLICATIONS PRESENTED IN HABILITATION THESIS

ERGOSTEROL AS AN EXAMPLE OF AN ORPHAN FUNGAL MAMP

Lochman, J., Mikes, V. Ergosterol treatment leads to the expression of a specific set of defence-related genes in tobacco (2006) *Plant Molecular Biology*, 62 (1-2), pp. 43-51

Contribution of author JL: Measurements of gene expression, WB analysis, Preparation of manuscript

Dadakova, K., Klemptova, J., Jendrisakova, T., **Lochman, J.**, Kasparovsky, T. Elucidation of signaling molecules involved in ergosterol perception in tobacco (2013) *Plant Physiology and Biochemistry*, 73, pp. 121-127

Contribution of author JL: Measurements of gene expression, Design of experiments, Preparation of relevant parts of manuscript

ELICITINS AS AN EXAMPLE OF A TYPICAL OOMYCETE MAMP

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8. SUPPLEMENTS

ERGOSTEROL AS AN EXAMPLE OF AN ORPHAN FUNGAL MAMP