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Biological effects of oomycetes elicitins

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Abstract: Successful plant defence responses to pathogen challenges are based on fast and specific pathogen recognition and plant reaction mechanisms. Elicitins, proteinaceous elicitors secreted by the *Phytophthora* and *Pythium* species, were first described in *Phytophthora* culture filtrates as proteins able to induce a hypersensitive response (HR) and resistance in tobacco at low concentrations. Later, they were classified as microbial-associated molecular patterns (MAMPs) able to induce defences in a variety of plant species. In this review, we present a comprehensive summary of the actual knowledge on the representative elicitins and their structure, perception and activation of plant signalling pathways. The current research of elicitins has been focused on a detailed understanding of the molecular mechanisms of the elicitin recognition by plant cells. Moreover, the possibility of elicitin involvement in the establishment and enhancement of plant host resistance to a broad spectrum of pathogens has been intensively studied.

Keywords: *Phytophthora*; cryptogein; infestin; plant immunity; resistance; pathogen

Extensive infections of agricultural crops by plant fungal and oomycete pathogens repeatedly have resulted in catastrophic harvest failures, thus, crop protection has constituted a major challenge of the past and present agricultural practise. Unlike animals, plants lack the adaptive immunity systems comprised of specialised mobile immune cells; however, they can efficiently activate a multilayer innate immune system eventually leading to plant resistance (Jones & Dangl 2006). Chemical compounds recognised by plants and able to elicit plant defence responses have been termed elicitors. A major part of plant defence responses against microbial pathogens depends on the effective detection of conserved microbial-associated molecular patterns (MAMPs) by membrane-localised pattern recognition receptors (PRRs) that induce a basal resistance response called MAMP-triggered immunity (MTI). Despite an increasing number of plant PRRs discovered over the past 20 years, for most of them, it has not been determined whether they can be recognized as true receptors thus far. This especially concerns receptor-like proteins, representing a parallel with the Toll-like receptors described in animal cells. In addition, an exposition to MAMPs can promote plants to a primed state of enhanced defences. This primed state is characterised by faster and stronger plant responses to a pathogenic stimulus compared to non-primed plants. A well-described model of plant-pathogen interactions is represented by the interactions of *Solanaceae* plants with proteinaceous elicitors secreted by oomycetes including many significant crop pathogens like *Phytophthora infestans* (Mont.) de Bary, the causal agent of late blight. Oomycete pathogens, on the other hand, also include the mycoparasite *Pythium oligandrum* Drechsler, used as a biocontrol agent against pathogenic fungi. Thus, understanding

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the mechanisms involved in the oomycete infection process can lead to the development of new methods providing increased and durable plant resistance.

Elicitins – proteinaceous elicitors of oomycetes. Oomycetes, fungus-like eukaryotic phytopathogens, which can exhibit biotrophic, necrotrophic or hemibiotrophic lifestyles, secrete elicitor proteins called elicitins. Elicitins are small proteins of about 10 kDa secreted by almost all the studied *Phytophthora* and some *Pythium* species (Fefeu *et al*. 1997; Panabieres *et al*. 1997). Elicitins are structurally highly conserved with no similarity to protein families in the plant kingdom (Jiang *et al*. 2006). The most studied elicitin-producing oomycetes include the hemibiotrophic *Phytophthora* spp. and some members of the necrotrophic *Pythium* spp.. Elicitins possess typical characteristic of MAMPs activating plant MTI and they have been suggested essential for an oomycetes life cycle as sterol transporters (Ponchet *et al*. 1999). On the other hand, their expression is down-regulated during the early biotic step of a *Phytophthora* infection (Attard *et al*. 2008; Colas *et al*. 2001) and they could constitute a class of elicitors in the middle of the evolutionary zigzag model (Derevnina *et al*. 2016).

Elicitins were divided according to the phylogenetic analysis into 4 elicitin (ELI) and 13 elicitin-like (ELL) clades. All the members of the ELI clades share a typical highly conserved 98-amino acid elicitin domain with at least 66% sequence identity and six cysteine residues at conserved positions forming three disulphide bridges (Figure 1A) (Boissy *et al*. 1996).

In the case of the ELL clades, the elicitin domain is more diverse at the sequence levels with various sequence spacing between the six cysteine residues (Jiang *et al*. 2006). Compared to elicitins from the ELI-1 clade composed uniquely by the elicitin domain, the elicitins from the other ELI clades possess an additional C-terminal domain of variable length and sequence, rich in threonine, serine and proline residues (Figure 1A) (Jiang *et al*. 2006). A potential *O*-glycosylation of this domain likely serves to anchor these elicitins to the cell wall, as proven for the glycoproteins POD-1 and POD-2 from *P. oligandrum* showing a typical elicitin signature and *O*-linked glycosylation sites (Takenaka *et al*. 2006).

During the last few decades, elicitins secreted during plant-oomycete interactions belonging to the ELI-1 clade have been extensively studied. The ELI-1 clade contains proteins with a conserved structure formed by five α-helixes, one β-antiparallel sheet and one ω-loop as in the case of β-CRY secreted by *Phytophthora cryptogea* Pethybr. & Laff. 1919 (Panabieres *et al*. 1997; Gooley *et al*. 1998). Inside the protein core, a hydrophobic cavity exists able to accommodate fatty acids and sterols (Mikeš *et al*. 1997). This class is further divided into two subclasses, ELI-1A (α-elicitins) and ELI-1B (β-elicitins), based on the isoelectric points of the corresponding elicitins (Figure 1). In general, basic β-elicitins display much stronger necrotic features in comparison to acidic α-elicitins, and also provide higher plant protection against any subsequent pathogen attacks.

Elicitin perception. A major part of the plant defences against microbial pathogens depends on the effective detection of MAMPs by the plasma membrane-localised PRRs that induce basal resistance responses within the MTI. However, the role of the receptor-like proteins (RLPs) in the MAMPs recognition by the plant cells is still poorly understood. Recently, a cell surface elicitin response RLP was characterised in the wild potato *Solanum microdontum* Bitter (Du *et al*. 2015). In this plant, the perception of elicitins was reported to depend on the receptor-like kinase (RLK) SERK3/BAK1, a major modulator of PRR-mediated immunity (Heese *et al*. 2007; Chaparro-Garcia *et al*. 2011). It was recently shown that ELR constitutively associates with a proposed general interactor for RLPs, the RLK SUPPRESSOR OF BIR1-1 (SOBIR1), which is known to be required for the INF1-triggered cell death in *Nicotiana benthamiana* Domin and *S. microdontum* (Domazakis *et al*. 2018). Elicitins recognition by ELR is likely mediated by the conserved structure of the elicitin domain, as suggested by observation that elicitins from different ELI clades, which exhibit relatively low sequence identity (Jiang *et al*. 2006), induced a hypersensitive response (HR) in transgenic potato plants expressing ELR (Du *et al*. 2015). The specific ω-loop region contains a highly conserved Leu41 residue important for the elicitin perception in different plant species (Dokládal *et al*. 2012; Starý *et al*. 2018).

Signalling events. Early events following the elicitin recognition have been only partially disclosed and include a typical immune response characterised by changes in the ion fluxes (Lecourieux-Ouaked *et al*. 2000) followed by the subsequent membrane depolarisation (Wendehenne *et al*. 2002), acidification of the cytoplasm and alkalisation of the extracellular space (Figure 2) (Pugin *et al*. 1997). Elicitins also trigger a burst of reactive oxygen species (ROS) and activation of the conserved pathogen-responsive MEK2-SIPK/

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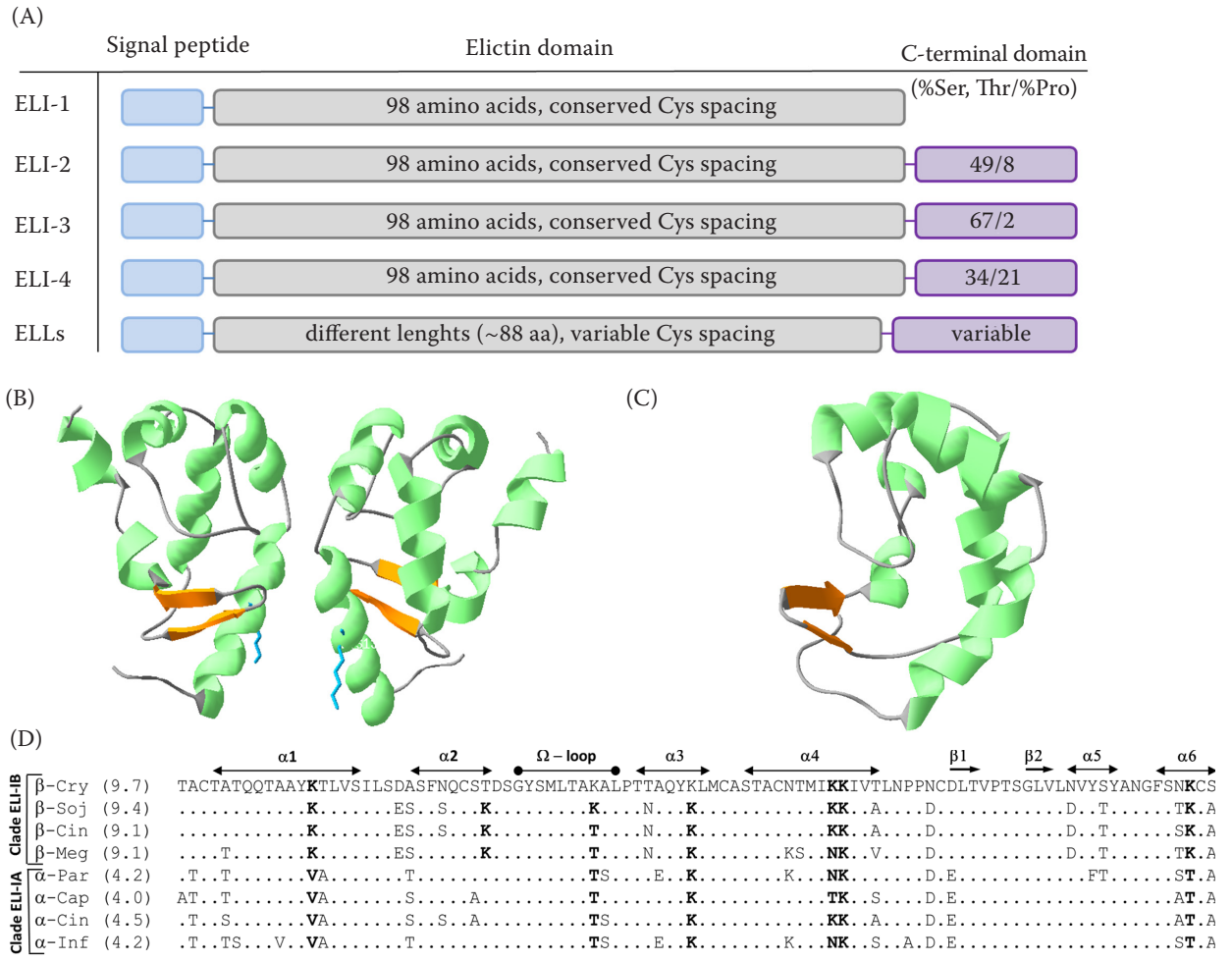

Figure 1. The classification and structures of the elicitin and elicitin-like proteins in oomycetes: (A) Structures of four ELI and ELL clades according to Jiang *et al.* (2006) showing the N-terminal signal peptide, the ELI domain and the variable C-terminal domain with a percentage representation of the Ser/Thr and Pro residues (Jiang *et al.* 2006), (B) the three-dimensional structures of the cinnamomin dimer, (C) the superimposition of β-CRY and the sylvaticin structures and (D) multiple sequence alignment of the 98 amino acid elicitin domain of ELI-1 from *Phytophthora* sp.

WIPK cascade mediated by the phosphorylation and up-regulation of several WRKY transcription factors (Xu *et al.* 2014; Starý *et al.* 2018). In tobacco, the plasma membrane NADPH oxidase, termed respiratory-burst oxidase homologue (RBOH), has a pivotal role in the first transient and the second sustained elicitin-induced ROS burst playing an important role in the regulation of the HR. The first rapid ROS burst is driven by the activation of the existing respiratory burst oxidase homologue protein D (RBOHD) proteins in the plasma membrane by the PRR-associated kinase BIK1 and calcium uptake; whereas the second massive ROS burst occurring hours after the elicitins perception is mediated by the prolonged activation of the MAPK activity, which is involved in the *de-novo* transcription of the RBOHD gene (Noirot *et al.* 2014).

Nitric oxide signalling participates in both the early and late phases of plant defence responses, including the HR and systemic acquired resistance (SAR). It has been recognised that nitric oxide (NO) involvement in the plant immune responses includes a cross-talk with the signalling pathways of ROS, involving S-nitrosation, a reversible posttranslational modification of the cysteine residues, which is considered to be a link between the redox changes occurring after the pathogen attack and the gene regulation of the defence proteins. The S-nitrosation of NADPH oxidase has been suggested as the crucial regulatory step for a pathogen-triggered oxidative burst (Yun *et al.* 2011). Nitrite reduction by the assimilatory nitrate reductase (NR, EC 1.7.1.1) is considered as the main NO source of elicitin-induced NO (Yamamoto-Katou *et al.*

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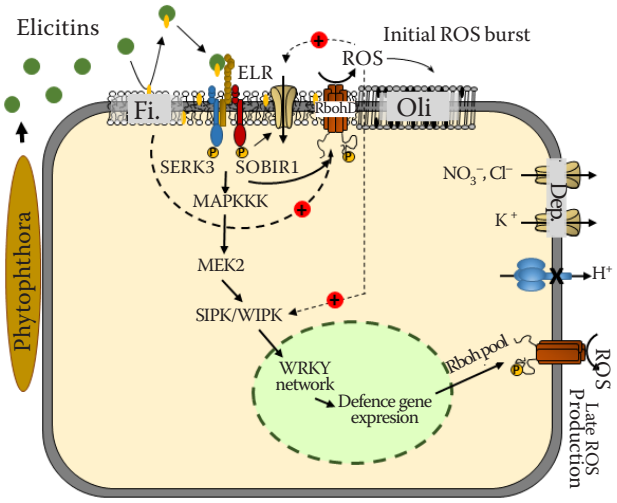

Figure 2. The signalling pathways activated by the oomycetes and elicitins with the highlighted early and late ROS bursts

cADPR – cyclic ADP ribose; E – effector; IP_3 – inositol trisphosphate; NO – nitric oxide; PK – protein kinase; RBOHA/B – respiratory burst oxidase homolog protein A/B; ROS – reactive oxygen species; SOD – superoxide dismutase; SIPK – salicylic acid-induced protein kinase; WIPK – wound-induced protein kinase; ELR – elicitin-response receptor; Fi. – fluidity increase; Oli. – order level increase; Dep. – depolarization

2006). NO production following β-CRY recognition is partly affected by the ROS, whereas NO regulates the intracellular levels of H_2O_2. Besides that, the rapid formation of peroxynitrite, the product of the reaction between NO and the superoxide radical, occurs in the elicited cells and is likely involved in the regulation of the plant responses to the elicitins (Kulik *et al.* 2015).

Elicitation also leads to changes in the intracellular trafficking. The β-CRY elicitin causes decreased levels of dynamins, proteins involved in cell trafficking, and increased levels of a specific 14-3-3 protein (Stanislas *et al.* 2009). This study proposed that plant micro-domains, similar to the lipid rafts present in animal cells, are involved in the early signalling steps during the elicitation. Moreover, the effects of the elicitins on the cellular trafficking during the plant defence responses, including clathrin-mediated endocytosis, were also described (Chaparro-Garcia *et al.* 2015). These processes ultimately lead to the up-regulation of the defence-related genes and to the establishment of the SAR against a wide spectrum of phytopathogens (Kamoun *et al.* 1993).

Plant responses to elicitins. Nowadays, recognised elicitin-responsive plant species include tomato, potato and pepper (*Solanaceae*), grapevine (*Vitaceae*), citrus (*Rutaceae*) and some radish and turnip cultivars (*Brassicaceae*) (Vleeshouwers *et al.* 2006; Kamoun *et al.* 1997; Dalio *et al.* 2017; Akino *et al.* 2014). However, elicitins induce HR cell death exclusively in plants of the *Nicotiana* genus and some *Solanum* spp. but not in other *Solanaceae* (Ponchet *et al.* 1999; Vleeshouwers *et al.* 2006). It seems obvious that the SAR induced by elicitins is derived from their presence mediated by the systemic movement across the plants (Devergne *et al.* 1992; Keller *et al.* 1996; Uhlíková *et al.* 2016). Initially, activation of the SA-mediated signalling pathway was considered the major signalling event in the elicitin-treated plants leading to induction of the SR, which can protect the host plant against the subsequent pathogen attacks (Ricci *et al.* 1989; Kamoun *et al.* 1993; Yu 1995; Keller *et al.* 1996). However, the treatment of different tomato genotypes with the INF1 (*P. infestans*) and β-CRY (*P. cryptogea*) elicitins or with elicitin-like proteins of the non-pathogenic organism *Pythium oligandrum* OLI and POD-1/POD-2 activates the jasmonic acid (JA) – and ethylene (ET) – mediated expression of the pathogenesis-related proteins. The elicitin treatment also induced a plant resistance against bacterial wilt disease, powdery mildew and *Phytophthora parasitica* Dastur, but not *P. infestans* (Picard *et al.* 2000; Benhamou *et al.* 2001; Kawamura *et al.* 2009; Satková *et al.* 2017; Starý *et al.* 2018). In summary, these findings suggest largely different signalling pathways in response to the elicitins across the plant taxa and probably reveal that other receptors, besides the recently described ELR in the potato, are involved in the elicitin perception in other plant species.

Features determining the ability to induce plant response. Elicitins were previously reported as a class of sterol carrier proteins and as small proteins able to cross the cell wall and transport sterols and fatty acids. This sterol-transporting ability has been generally considered as their primary function as the *Phytophthora* and *Pythium* species are not able to synthesise the sterols necessary for their life cycle and are completely reliant on external sterol sources (Hendrix 1970). After the sterol binds into the elicitin cavity, a shift in the ω-loop conformation of β-CRY is observed (Boissy *et al.* 1999), which has been proposed to play a substantial role in the elicitin-inducted plant resistance (Osman *et al.* 2001). Nevertheless, construction of elicitin mutants impaired in the sterol binding showed that

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this is not the crucial factor determining the defence elicitation capabilities of elicitins. Rather, the sterol binding enhances the fluidity of the plant plasma membrane, which seems to be another key point in the early signalling steps and induced ROS production (Dokládal *et al*. 2012; Sandor *et al*. 2016).

In recent studies, the important role of the individual amino acid residues responsible for the global charge of elicitins on the ability to induce HR-cell death and the SAR has been suggested. A specific role in the HR-inducing capacity was repeatedly demonstrated for the residue in the position 13 of the elicitin domain (O'Donohue *et al*. 1995; Plešková *et al*. 2011). The neutral amino acid valine is found in this position in the acidic α-elicitins, which require micro-molar concentrations to induce the necrosis in tobacco leaves or tobacco cells. In comparison, basic β-elicitins with the amino acid lysine in this position already induce strong necrosis at nano-molar concentrations (Figure 1). Moreover, a mutation of lysine 13 for valine in β-CRY was found to change its behaviour as a basic elicitin to an acidic one (Plešková *et al*. 2011). Uhlíková *et al*. (2016) systematically replaced other lysine residues of β-CRY by threonine and also described the important role for residue lysine 39 involved in the induction of the necrosis and plant resistance (Figure 1). Principally, this study uncovered that the biological activity of elicitins, in terms of the SAR induction, results from a combination of several factors including the overall surface charge, the presence of specific lysine residues or the capacity to interact with other endogenous plant partners. Interestingly, plant lipid transfer proteins, sharing certain structural and functional similarities with elicitins, have been suggested as their potential interacting partners, the endogenous (Blein *et al*. 2002), when recently nsLTP1-elicitin (non-specific lipid transfer proteins) complex formation curves were measured by QCM driven by a counter UZ 2400 (Grundig, Germany) (Uhlíková *et al*. 2016).

Early studies also proposed that the formation of elicitin homodimers might be a crucial point of the biological activity of elicitins (Ponchet *et al*. 1999). Recently, Uhlíková *et al*. (2016) brought new evidence supporting this hypothesis by the characterisation of the kinetic parameters of the elicitin homodimer formation and the demonstration of the key role of the specific lysine residues for the dimer formation and the SAR induction of SAR in distal plant leaves. Nevertheless, the exact role of the dimer formation in the biological activity of elicitins remains to be elucidated upon.

CONCLUSIONS

Elicitins and their effects on plant immunity have been extensively studied since their discovery in 1980s; however multiple components involved in their perception and signal transduction within the plant cells still have not been completely characterised. Studies of plant response to elicitins established an excellent model system of plant–pathogen interactions to uncover the signalling pathways, which may lead to the establishment of host resistance. Currently, the elicitin research is focused on the identification and characterisation of the cell surface receptors responsible for the elicitin recognition (Chaparro-Garcia *et al*. 2011; Du *et al*. 2015; Peng *et al*. 2015) and on the characterization of the molecular mechanisms of the plant responses to the elicitins (Jiang *et al*. 2006). Recent studies have been focused on the detailed characterisation of the molecular mechanisms involved in the priming of the plant defences. Chromatin modifications were proposed to affect the priming of the defence genes through faster and stronger transcription, represented by the reported involvement of histone deacetylases as negative regulators of the elicitor-induced cell death in tobacco (Bourque *et al*. 2011). Besides this, the utilisation of elicitins to achieve an increased resistance in agricultural crops against a wide spectrum of microbial pathogens is being explored (Du *et al*. 2015; Ouyang *et al*. 2015).

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