Plant Physiology and Biochemistry 97 (2015) 82–95

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

The effect of cryptogein with changed abilities to transfer sterols and altered charge distribution on extracellular alkalinization, ROS and NO generation, lipid peroxidation and LOX gene transcription in *Nicotiana tabacum*

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ARTICLE INFO

Article history:
Received 14 May 2015
Received in revised form
15 September 2015
Accepted 15 September 2015
Available online 21 September 2015

Keywords:
Cryptogein
Hydroxy fatty acids analysis
Hypersensitive response
Lipid peroxidation
Mutant proteins
Nicotiana tabaccum

ABSTRACT

Cryptogein, a protein from oomycete *Phytophthora cryptogea*, induces a hypersensitive cell death in *Nicotiana tabacum*. We prepared a new series of cryptogein mutant proteins with altered abilities to bind sterols and with altered charge distribution in the proteins. The effect of the mutations on the cryptogein ability to induce plant defence mechanisms associated with hypersensitive cell death were examined. Our results with new mutants support the previous findings that the sterol binding does not influence synthesis of ROS, cytosol acidification and development of leaf necrosis as these events seem to be more likely affected by the charge distribution and the overall protein structure. This hypothesis was also applicable on other mechanisms involved in the execution of plant cell death such as the NO generation, the stimulation of lipid peroxidation (determination of malondialdehyde and hydroxy fatty acids levels) and LOX gene transcription. In addition, the ability to bind sterols was found to serve not only for pathogen utilisation in its own metabolism but also to have an important function for the destabilization of plant membrane facilitating the pathogen spread inside the plant tissue as well as intensively contributing to the development of plant cell death. Considering the insertion of charged amino acid residues in the protein structure, the change localized in the protein surface affected its biological activity more effectively than that change inside the protein cavity. Moreover, the insertion of negative charged amino acids influenced mainly the events involved in the early phase of defence reaction, while the positive residues affected especially the necrotic activity of cryptogein.

1. Introduction

Plants are frequently challenged by potential pathogens and have therefore evolved inducible defence mechanisms to survive in their environment. A key difference between resistant and susceptible plants is the timely recognition of the invading pathogen and the rapid and effective activation of host defence. This recognition is mediated by an interaction between specific plant receptors PRR (pattern-recognition receptors) and corresponding molecules such as microbial-, pathogen- or damage-associated molecular patterns (MAMPs, PAMPs or DAMPs) formerly known as elicitors. (Jones and Dangl, 2006).

One of the frequently studied model of incompatible interactions is the interaction of tobacco (*Nicotiana tabacum*) with cryptogein, a proteinaceous elicitin secreted by oomycete

Abbreviations: 9-HODE, 9-hydroxy-10,12(Z,E)-octadecadienoic acid; 9-HOTE, 9-hydroxy-10,12,15(E,Z,Z)-octadecatrienoic acid; 12-HOTE, 12-hydroxy-9,13,15(Z,E,Z)-octadecatrienoic acid; 13-HODE, 13-hydroxy-9,11(Z,E)-octadecadienoic acid; 13-HOTE, 13-hydroxy-9,11,15(Z,E,Z)-octadecatrienoic acid; 15-HEDE, 15-hydroxy-11,13(Z,E)-eicosadienoic acid; 16-HOTE, 16-hydroxy-9,12,14(Z,Z,E)-octadecatrienoic acid; 18:2, linoleic acid; 18:3, α-linolenic acid; CaM, calmodulin; CDPKs, Ca^{2+}-dependent protein kinases; DAF-FM DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DHE, dehydroergosterol; FW, fresh weight; HFA, hydrox fatty acid; HPLC, high-pressure liquid chromatography; HR, hypersensitive reaction; LP, lipid peroxidation; LOX, lipoxygenase; MAPKs, mitogene-activated protein kinases; MDA, malondialdehyded; NO, nitric oxide; $ONOO^-$, peroxynitrite; PCD, programmed cell death; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; TBA, thiobarbituric acid; TEP, 1,1,3,3-tetraethoxypropane; X24, recombinant cryptogein.

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http://dx.doi.org/10.1016/j.plaphy.2015.09.009
0981-9428/

Phytophthora cryptogea. Cryptogein is composed of 98 amino acid residues with molecular weight 10 kDa (Ricci et al., 1989). This protein induces an intense defence response in tobacco consisting of a hypersensitive reaction (HR) that is characterized by the programmed cell death (PCD) at the site of infection and the development of a systemic acquired resistance (SAR) (Rustérucci et al., 1999; Dokládal et al., 2012).

The present paper is focused on the pathways of the plant defence response that are connected with HR cell death. Lipid peroxidation (LP) is usually used as a marker of stress situations and is often associated with plant cell death as it occurs at the same time as the appearance of leaf necrosis (Montillet et al., 2002; Rustérucci et al., 1999). LP is a process of fatty acid oxygenation that leads to the degradation of biological membranes and, consequently, to the loss of membrane integrity and functions, i.e. the basic character of living cells. In addition, LP generates hydroperoxides of polyunsaturated fatty acids (PUFAs) as primary products that are further converted to a range of secondary products such as ROS, lipid radicals, aldehydes, alkanes etc. These secondary products further contribute to the plant defence response as signalling compounds or by their antimicrobial activity or their involvement in the induction of defence gene expression (Prost et al., 2005; Thoma et al., 2003). LP can be mediated nonenzymatically by the action of free radicals, among reactive oxygen species (ROS) or nitric oxide (NO) can belong, or enzymatically by the lipoxygenase (LOX) activity (Rustérucci et al., 1999).

ROS and NO are key signalling molecules produced in the early phase of defence response and they are involved in elicitor-transduction cascades leading to both the HR cell death of plant tissue and SAR as reviewed in Garcia-Brugger et al. (2006).

The major enzymes responsible for a rapid generation of a huge amount of ROS in cryptogein-induced HR are NADPH oxidases that catalyse the production of superoxide anion, a precursor for the generation of H_2O_2 and other ROS (Simon-Plas et al., 2002). NO can be synthetized through both nonenzymatic and enzymatic reactions as by the activity of nitrite reductase (del Río et al., 2004; Yamamoto et al., 2003). Recent studies also suggested the existence of arginine-dependent NO production in plants, an enzymatic activity similar to the animal NO-synthase (NOS). The gene encoding NO-synthase and the corresponding protein has not been found in plants yet, however, there is a possibility that the NOS-like activity could be carried out by several proteins functioning together to generate NO from arginine using the same substrate and cofactors as the animal NOS (Corpas et al., 2006, 2009).

A significant cross-regulation between NO and ROS production in plants following pathogen infection has been found (e.i. inactivation of NADPH-oxidase by S-nitrosylation), together with a cross-talk in NO- and ROS-mediated signalling pathways. ROS such as H_2O_2 and NO act either independently or in partnership in the regulation of gene expression and in the execution of hypersensitive cell death. NO and H_2O_2, individually, can also affect the same signalling pathways with similar downstream responses. Some common intermediates of both ROS and NO signalling pathways are Ca^{2+} and Ca^{2+}-binding proteins, such as CaM. On the other hand, the generation of cyclic GMP seems to be NO-dependent, whereas activation of G proteins is attributed to ROS signalling (Asai et al., 2008; Garcia-Brugger et al., 2006; Oda et al., 2010). It was described that the activation of plant cell death during HR results from a combined interactions between NO and H_2O_2. NO involvement in HR definitively requires H_2O_2 but by itself it is not effective in triggering of HR cell death. (Zago et al., 2006).

The plant LOXs, non-hem iron-containing compounds, are classified into two groups according to their regio-specific dioxygenation of PUFAs; 9-/13-LOXs generating 9-/13-hydroperoxy PUFA. Cryptogein induced LP is mediated by an intense activity of 9-LOX and low free radical-dependent action. 13-LOX-mediated LP is constitutively present in a low level. (Rustérucci et al., 1999). The method for the discrimination of 9-LOX, 13-LOX and radical-mediated LP has been previously described (Degousée et al., 1994; Rustérucci et al., 1999). This method is based on the determination of the different primary compounds resulting from the enzymatic and nonenzymatic LP which can be identified and quantified on the basis of their regio- and stereo-specifity. In this paper, the effect of altered cryptogein structure on the individual pathways of LP was examined.

Previous studies show that cryptogein together with other elicitins are a new class of sterol carrier proteins which are able to bind and transport sterols between biological membranes. Additionally, elicitins are known to bind also fatty acids although this affinity is significantly lower. (Dobeš et al., 2004; Vauthrin et al., 1999) Both sterols and fatty acids are bound into hydrophobic cavity located in the elicitin core and this binding was assumed to be essential for cryptogein attachment to the specific plant receptor and so for its biological activity in term of ROS production and plant cell death induction (Hirasawa et al., 2004). To verify this theory, the series of cryptogein mutant proteins with limited ability to bind sterols and fatty acids were constructed by site-directed mutagenesis of chosen amino acids located in the hydrofobic cavity (Lochman et al., 2005). Mutant variants L19R and L15W/L36F were prepared and their structures were tested by far-UV-CD spectroscopy, the mutations did not perturb markedly their structure as compared to cryptogein. However, in protein L15W/L36F the mutation resulted in a conformation change in ω loop very similar to that observed by binding of sterols which could be considered as a one of important parameters for cryptogein binding to plant receptor and also for its biological activity. The effects of expressed mutant proteins on the external alkalization, ROS production, induction of HR cell death and the expression of selected defence genes in tobacco cells or tobacco plants compared to that of wild type cryptogein were evaluated (Lochman et al., 2005). The results and results of Hirasawa et al. (2004) suggest that the ability to induce the early events could be conditioned by the conformation change of the ω loop induced by the sterol binding or by a mutation whereas the ability to express PR proteins and to induce HR cell death is by overall structure of the proteins and charge distribution. However, the tested mutant proteins were expressed in *Pichia pastoris* expression system and at the N-terminus, there was the EAEA amino acid sequence due to an incomplete cleavage of signal peptide caused by *P. pastoris*. This peptide gave a higher negative charge to N-terminus of expressed proteins and it could then influence the biological activity of the mutant proteins, therefore, the presented findings are suggested to be artifacts due to the additional amino acid sequence. As a consequence, the mutant proteins without EAEA sequence were prepared and their biological activity was analysed.

On the basis of results obtained from cryptogein mutant proteins with EAEA sequence (Lochman et al., 2005), the protein L19R and L15W/L36F without EAEA sequence were prepared. In the present work, the effect of these proteins on both the early and late signalling pathways associated to the development of plant HR cell death was evaluated. The stimulation of the ROS and NO synthesis and pH changes in tobacco cells together with the ability of the individual proteins to induce 9-LOX gene expression and LP (necrotic effects) in tobacco plants were determined and compared with the activities of cryptogein (recombinant protein X24) and the protein L19R with EAEA sequence. In addition, the influence of one arginine residue which was spontaneously added by *P. pastoris* at the N-terminus of protein L15W/L36F on its biological activity was examined since this protein variant displayed interesting results able to show the impact of the positive charge addition in the