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REVIEW

Participation of Proteolytic Enzymes in the Interaction of Plants with Phytopathogenic Microorganisms

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Abstract—Different forms of participation of proteolytic enzymes in pathogenesis and plant defense are reviewed. Together with extracellular proteinases, phytopathogenic microorganisms produce specific effectors with proteolytic activity and are able to act on proteins inside the plant cell. In turn, plants use both extracellular and intracellular proteinases for defense against phytopathogenic microorganisms. Among the latter, a special role belongs to vacuolar processing enzymes (legumains), which perform the function of caspases in the plant cell.

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Plant genomes contain a multitude of genes coding for proteolytic enzymes of different catalytic types. The number of such genes identified in *Arabidopsis thaliana* is close to 500. The most widely represented proteins are enzymes from the subtilisin (S8), papain (C1), and pepsin (A1) families [1]. Such a complex set of proteinases reflects their functional diversity in plants. Proteolytic enzymes not only catalyze the degradation and posttranslational conversion of storage proteins in plant seeds [2-4], but also play a crucial role in the regulation of such important physiological processes as aging of leaves and flowers, formation of conduction pathways, embryogenesis, and a number of others [5-7]. Proteinases also play an active role in plant response to various external stimuli, both of abiotic and biotic nature. The latter includes the interaction of plants with pathogenic microorganisms [8]. In turn, phytopathogenic microorganisms widely use proteolytic enzymes for penetration into the host plant and obtaining nutrients, as well as for active effect on plant protection barriers [9-11].

The aim of the present review is to consider the available data regarding the different forms of participation of proteolytic enzymes in the interaction of plants with microorganisms.

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PROTEINASES FROM MICROORGANISMS

Phytopathogenic bacteria and fungi secrete into their medium a large set of hydrolases, which play an active role in the permeation of plant defense barrier and progression of diseases [12]. These proteins include proteolytic enzymes of different catalytic types [9]. The important role of proteinases is indicated by direct dependence (observed in a number of cases) between the activity of extracellular proteinases in microorganisms and intensity of plant disease [13-16]. The same conclusion follows from many experiments showing that protein inhibitors of proteinases (contained in plants) not only suppress the activity of enzymes secreted by microorganisms, but also inhibit their growth and developmental effects [17-20]. It was recently shown that transgenic expression of proteinase inhibitors in plants promotes their resistance to bacterial and fungal infections [21]. The participation of extracellular microbial proteinases in pathogenesis can be of different nature: from degradation of cell wall proteins [14, 22] and other defense proteins in plants [23, 24] to processing of inherent extracellular proteins of microorganisms, which are significant for progression of a disease [25]. The effect of extracellular proteinases from phytopathogenic microorganisms was discussed in detail in our previous reviews [9, 26, 27].

However, the participation of proteinases in pathogenesis is not limited by the enzymes excreted by microorganisms in the environment. There are mechanisms allowing for protein transfer from bacterial cell across the membrane and cell walls directly into the apoplast or cytoplasm of a plant cell. One of these mechanisms is a type III secretion system (T3SS) found in Gram-negative bacteria [28, 29]. The proteins transferred in this way (effectors) are the products of avirulence genes (*Avr*) able to cause the defense reaction in plants containing the corresponding resistance genes (*R*). At the same time, they facilitate the progression of a disease in plants lacking the *R*-genes [30]. Gram-negative bacteria with T3SS include many plant pathogens belonging to genera *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia*, and *Pantoea* [31]. The first results of the study of biochemical mechanisms underlying the action of the effector proteins were obtained after it was determined that a number of them display enzymatic (including proteolytic) activities [32]. The effector proteins known to date as proteinases are presented in the table. They can be divided into four protein families. The members of the YopJ and XopD families belong to the CE clan of cysteine proteinases, whereas the members of YopT and AvrRpt2 belong to the CA clan. The properties of a number of effectors are reviewed in detail below.

The avirulent protein AvrPphB from the bacterium *Pseudomonas syringae* pv. *phaseolicola* with a molecular weight of 35 kD undergoes autoactivation in the bacterial cell with the formation of two fragments, 7 and 28 kD. After transfer into a plant cell, the 28 kD fragment is acylated by a fatty acid residue and transferred into the cytoplasmic membrane. AvrPphB causes a hypersensitive response in *Arabidopsis* plant expressing RPS5 protein [11]. By its three-dimensional structure and location of Cys98, His212, and Asp227 residues (forming the catalytic triad of the proteinase active site) AvrPphB resembles papain [47]. Mutants with replaced Cys, His, and Asp residues in the catalytic site equally lost their biological activity and ability for autoactivation [38, 39]. The inter-

Bacterial effector proteins displaying the properties of cysteine proteinases

T3SS family	Effector	Microorganism	Proteinase family*	Substrate	Reference
YopJ	AvrXv4, AvrBsT, AvrRxv, XopJ	*X. campestris* pv. *vesicatoria*	C55	SUMO–protein conjugates	[33]
	PopP1, PopP2, PopP3	*Ralstonia solanacearum*	C55	*Arabidopsis* RRS1 protein	[34]
	AvrPpi G1	*Ps. syringae* pv. *pisi*	C55		[35]
	HopPma D	*Ps. syringae* pv. *maculicola*			[36]
	ORF B	*Erwinia pyrifoliae*			[11]
	Y410	*Rhizobium* sp. NGR234			[11]
XopD	XopD	*X. campestris* pv. *vesicatoria*	C48	SUMO–protein conjugates	[37]
	PsvA	*Ps. syringae* pv. *eriobotryae*			[11]
YopT	AvrPph B	*Ps. syringae* pv. *phaseolicola*	C58	*Arabidopsis* PBS1 protein	[38, 39]
	AvrPpi C2	*Ps. syringae* pv. *pisi*			[35]
	HopPto C, HopPto N	*Ps. syringae* pv. *tomato*	C72		[40, 41]
	BLR2140, BLR2058	*Bradyrhizobium japonicum*			[42, 43]
	Y4z C	*Rhizobium* sp. NGR234			[44]
AvrRpt 2	AvrRpt 2	*Ps. syringae* pv. *tomato*	C70	*Arabidopsis* RIN4 protein	[45]

* Here and further below the MEROPS database is used for proteinase classification [46].

action of AvrPphB with RPS5 is not direct. The presence of a third component, PBS1 protein (a true substrate for proteinases), is necessary. PBS1 protein, which displays the properties of serine/threonine kinase, is cleaved in two fragments, then undergoes autophosphorylation and acts like a trigger activating the signal transduction mediated by RPS5 [11, 39]. It has been assumed that all the three proteins involved in the process remain associated with the plasma membrane [48].

Another effector, AvrRpt2, found in *Ps. syringae* pv. *tomato*, belongs to a distinct family and has no analogs so far (see table). AvrRpt2 induces a hypersensitive response in resistant *Arabidopsis* plants expressing the RPS2 protein. On the contrary, in plants lacking this protein, AvrRpt2 effector facilitates the progression of disease [10]. The effector protein with a molecular weight of 28 kD undergoes proteolytic cleavage inside the plant cell at the Gly71–Gly72 bond, resulting in the formation of two fragments of 21 and 7 kD. The C-terminal fragment (21 kD) acts as a trigger of a defense response [49]. Despite the fact that the processing of AvrRpt2 is an autocatalytic process, the presence of some auxiliary protein factor, contained in extracts of animal and plant tissues, is necessary [50]. The structure of the AvrRpt2 effector molecule and the location of catalytically active amino acid residues have a certain similarity to staphopain, a cysteine proteinase from *Staphylococcus aureus* [45]. The RIN4 protein was identified as a substrate for AvrRpt2 in *Arabidopsis* cells [51, 52]. RIN4 proteolysis is a signal for hypersensitive response, where RPS2 is also involved. However, purified AvrRpt2 effector has no effect on RIN4 *in vitro*. The interaction requires the presence of a eukaryotic cofactor, which is apparently identical to that involved in the processing of the effector itself [45]. The nature of this cofactor has been recently established. It was identified as a cyclophilin protein [53].

Different bacteria of the *Yersinia* genus, which are animal and plant pathogens, produce effectors known as Yop (*Yersinia* outer proteins). Being introduced into a host cell (animal or plant), they suppress the elicitation of the immune response [54]. Some of the effectors, such as AvrBsT and AvrXv4 from *Xanthomonas campestris* pv. *vesicatoria*, belonging to the YopJ protein family, display a significant similarity to proteinase ULP1 from the yeast *Saccharomyces cerevisiae* [33, 55]. The ULP1 enzyme regulates the interaction of SUMO (small ubiquitin-like modifier) with different proteins in yeast [56]. Proteinases of this type contain the triad His/Glu/Cys or His/Asp/Cys in their catalytic site and belong to the CE clan of cysteine proteinases [55]. Modification of the residues in the enzyme active site can result in the loss of its ability to elicit hypersensitive response in plants [33, 37, 55].

Expression of *X. campestris* effectors, XopD and AvrXv4, in *Nicotiana benthamiana* (L.) leaves results in decrease in content of SUMO–protein conjugates. On this basis, it was assumed that the effectors have real isopeptidase activity *in vivo* [33, 37]. This was further confirmed using purified XopD protein. It was demonstrated that the effector acts as isopeptidase *in vitro*, cleaving the bond between the carboxyl group of N-terminal SUMO residue and ε-amino group of Lys residue in the protein substrate. Moreover, it can act as a peptidase able to convert the SUMO precursor into its active form, containing the Gly-Gly sequence at the N-terminus [37, 57]. The action of XopD is highly specific, and its activity is displayed entirely towards the SUMO forms of plant origin [37].

Modification of SUMO proteins plays an important role in the regulation of various biological processes in plants. In this case, according to available data, the proteins being modified are mainly localized in the nucleus [58]. In this connection, it is noteworthy that the XopD and PopP2 effectors entering the plant cell are also localized in nuclei [34, 37]. It can be assumed that the presence of proteinases able to imitate the action of cognate plant enzymes provides the phytopathogenic microorganisms with important advantages. In turn, mechanisms may have evolved in plants that can neutralize the activity of bacterial SUMO proteinases. The product of a resistance gene in *Arabidopsis*, RRS1 protein, provides resistance towards *Ralstonia solanacearum* strains, which express PopP2 effector [59]. Moreover RRS1 protein directly interacts with bacterial proteinase [34].

Products of avirulence genes exhibiting proteolytic activity were also found in phytopathogenic fungi. The most destructive pathogen of rice (*Oryza sativa* L.), the fungus *Magnaporthe grisea*, produces the protein Avr-Pita, which, being a proteinase, belongs to the family of zinc-containing proteinases M35 [60]. The mature form of the enzyme can induce a hypersensitive response in rice. It was determined that Avr-Pita directly interacts with a leucine-rich domain of the product of the resistance gene, the Pi-ta protein [61]. The mutant form of Avr-Pita proteinase, where the Glu residue in the active site is replaced by an Asp residue, is unable to interact with the Pi-ta protein and, accordingly, has no biological activity [62]. The data led to the conclusion that the product of a resistance gene, the Pi-ta protein, is itself the substrate for the proteinase. This is the first case of direct interaction between the products of avirulence and resistance genes described in the literature [10].

These data indicate that posttranslational processing of proteins directly inside the plant cell can be important for the effect of phytopathogenic microorganisms on defense systems in plants [10, 11, 30].

PLANT PROTEINASES

Intracellular proteinases. The usual defense reaction in plants upon their interaction with pathogenic microor-

ganisms is a hypersensitive response, including programmed cell death (PCD) in injury sites [12, 63, 64]. The cell death disturbs normal nutrition of the pathogen and prevents invasion of healthy tissues. PCD can even release the signal inducing the defense reaction in surrounding healthy cells and in the entire plant [65].

In spite of the general similarity between PCD in plants and apoptosis in animals, there are, however, certain differences, associated (among other things) with the peculiarities of the structure of plant cells and the absence of phagocytosis in plants [66-68]. Despite the fact that ideas concerning PCD in plants were established much earlier than apoptosis in animals was described [69], molecular mechanisms of PCD in plants are less studied. This is also true for proteinases regulating the PCD process in plants.

Upon apoptosis in animals, the key role is played by caspases, cysteine proteinases cleaving the peptide bonds formed by the carboxyl group of Asp residues [70, 71]. The first results clarifying the nature of proteinases responsible for PCD in plants were obtained using specific caspase inhibitors. Such inhibitors include fluoro/chloromethylketones (CMK) or aldehydes (CHO) of peptides containing a short amino acid sequence recognized by caspases. It was demonstrated that chloromethylketone Ac-YVAD-CMK and aldehyde Ac-DEVD-CHO, being inhibitors of caspase-1 and caspase-3, respectively, slow the PCD process in tobacco (*Nicotiana tabacum* L.) upon its interaction with avirulent pathogen *Ps. syringae* pv. *phaseolicola* or tobacco mosaic virus (TMV) [72]. The number of such examples is rather large [8]. It was recently shown that VirD2 protein from *Agrobacterium tumefaciens* is cleaved by human caspase-3 and proteinase from the leaves of tobacco plants infected with TMV at two identical peptide bonds. The inhibitor constructed based on one of the two cleaved amino acid sequences, biotinyl-TATD-CHO, not only suppressed the activity of tobacco proteinase *in vitro*, but also blocked the PCD process *in vivo* [73]. When evaluating all these data, it should be noted that the inhibitors of caspases of this type also affect cysteine proteinases belonging to papain (C1) and legumain (C13) families [74]. The presence of enzymes with caspase-like activity in plants was additionally confirmed by experiments with transgenic tobacco plants containing the p35 gene from baculovirus, a broad range caspase inhibitor. Expression of p35 suppresses a hypersensitive response and results in a decrease in plant resistance to TMV. In this case, the effect was directly dependent on the presence of caspase-hydrolyzed peptide bond in p35 [75, 76].

Despite the clear evidence of the presence of enzymes in plants with activity similar to that of animal caspases, the genes of plant caspases as such have not been identified so far [77]. The data obtained in recent years led to the conclusion that caspase functions in PCD process in plants can be carried out by vacuolar processing enzymes (VPE, legumains) [78-80]. Like caspases, VPE or legumains belong to the CD clan of cysteine proteinases, within which they form the separate family C13 [81]. Despite the fact that the homology between caspases and legumains is relatively low, there is a noticeable similarity in the structure of their active sites and overall spatial structure [82]. Initially, plant legumains were known as enzymes playing an active role in mobilization of storage proteins in seeds upon their germination [2, 4]. Later it was established that legumains can take part in processing of storage proteins as well as some other vacuolar proteins in plants [3, 83-85]. They are capable of autoactivation at slightly acidic pH typical for the inner space of vacuoles [86]. Like caspases, VPE can hydrolyze peptide bonds containing Asp residue in the P1 position, however with lower efficiency than those containing Asn residue in this position [87, 88]. Moreover, the activity of VPE from *Nicotiana benthamiana* (L.) was suppressed by the caspase-1 inhibitor Ac-YVAD-CHO [89].

Recently it was demonstrated that the content of mRNA and VPE protein in *N. benthamiana* leaves increases rapidly upon infection with TMV. Deficit in VPE results in the loss of PCD ability [89]. Expression of one of the four VPE (VPEγ) forms in *Arabidopsis* was intensified upon infection with avirulent *Ps. syringae* pv. *tomato* DC 3000 strain [90]. An analogical effect was observed on plant infection with *Botrytis cinerea* fungus and turnip mosaic virus [90]. These data are in agreement with the results of previous investigations, which have demonstrated that disintegration and collapse of vacuolar membranes results in the degradation of cytoplasm structures and death of plant cells [69]. According to the suggested model, VPE activation in response to different stress stimuli triggers the activation of other hydrolases (proteinases, nucleases, lipases) localized in vacuoles. In turn, this leads to tonoplast destruction, rapid degradation of cell content and, ultimately, to cell death [91]. Full degradation of DNA in the nucleus and in chloroplasts in the process of xylogenesis in *Zinnia elegans* L. cells occurs during the first 15 min after the rupture of the tonoplast and collapse of vacuoles [92].

Another group of proteinases, which might be closely associated with induction of PCD in plants, is the metacaspases [76, 80]. Metacaspases are similar to caspases in their primary structure and spatial conformation [93]. In plants they are represented by two types of enzymes, metacaspases I and II. Metacaspases I contain N-terminal prodomain, which is absent in metacaspases II [76]. Contrary to VPE (and similar to animal caspases), metacaspases II are localized in the cytosol [80]. The content of metacaspase II mRNA rapidly increased in tomato leaves in the process of PCD caused by infection with fungal pathogen *B. cinerea* [94]. Mutational analysis has demonstrated that metacaspase ability to induce PCD in *Arabidopsis* depends on the presence of cysteine residue in the enzyme active site [95]. The study of substrate

specificity of two metacaspases, AtMCP1b and AtMCP2b, has shown that they (contrary to caspases) predominantly cleave peptide bonds containing residues of basic amino acids (such as Arg and Lys) at the P1 position [95]. Other *Arabidopsis* metacaspases II display similar substrate specificity [96]. At the same time, metacaspase II playing an active role in PCD during embryogenesis of Norwegian spruce (*Picea abies* L. Karst.) cleaved the peptide bonds formed by the carboxyl group of Asp residues. By the nature of its effect on substrates and towards inhibitors, this enzyme (VEIDase) resembles animal caspase 6 [97].

It may be that the caspase-like effect can be exhibited by not only cysteine proteinases, but also by enzymes belonging to other catalytic types. It is known that the treatment of oat (*Avena sativa* L.) with victorin (a toxin from the phytopathogenic fungus *Cochliobolus victoriae*) induces an apoptosis-resembling PCD [98]. Two serine proteinases, SAS1 and SAS2, were isolated from the leaves of such plants, both belonging to the subtilisin family (S8). The enzymes cleave peptide bonds where an Asp residue is located at the P1 position, and their activity can be blocked by caspase inhibitors. In this connection, the name "saspases" was suggested for these proteinases [99]. The characteristic feature of saspases is that they are constitutively expressed in cells and are secreted in active form in the intercellular space during the early stages of PCD [99].

Extracellular proteinases. Plant apoplast is characterized by high content of hydrolytic enzymes including proteinases of different catalytic types: serine [100, 101], aspartic [102, 103], cysteine [104], and metal-containing enzymes [105]. Extracellular proteinases can play an important role in plant defense against phytopathogenic microorganisms [106]. This is indicated, in particular, by the observed increase in proteinase secretion in response to plant infection with pathogenic microorganisms [103, 107].

One of the most studied apoplast enzymes is serine proteinase P69, which was first found in tomato leaves (*Lycopersicon esculentum* Mill.) affected by viroid [100, 101]. P69 mRNA is accumulated in large quantities in extracellular space in leaves and stems of the infected plant and is completely absent in healthy plants [108]. By its properties, the enzyme is similar to bacterial subtilisins and belongs to subfamily S8A of serine proteinases [1]. Genetic analysis has demonstrated that tomato proteinase P69 is occurred in several forms, two of which (P69B and P69C) are expressed in response to plant infection with pathogenic microorganisms, as well as upon treatment with salicylic acid [109-111]. Proteinases with similar properties have been found in *Arabidopsis* [1, 112]. The similarity between the properties of plant subtilases and animal proprotein convertases suggests that they are actively involved in protein processing and release of physiologically active peptides [109, 113, 114]. It was found that tomato proteinase P69B is involved in processing of a leucine-rich protein of the extracellular matrix [115]. It is known that many proteins of this type are products of resistance genes and have a key role in recognition of T3SS effectors and induction of defense response [11]. Confirmation of the important role of plant serine proteinases in interaction with pathogenic microorganisms is production of specific serine proteinase inhibitors by some pathogens. Thus, the oomycete *Phytophthora infestans*, the major potato and tomato pathogen, secretes (in the apoplast) proteins acting as inhibitors of the above P69 proteinase and subtilisin A; however, these proteins are not active towards animal serine proteinases and chymotrypsin [116, 117].

Along with serine proteinases, an aspartic proteinase with molecular weight of 37 kD and pH optimum of activity of 2.5-3.5 was found in the extracellular space of tomato leaves infected by viroid [102]. A proteinase with similar properties was found in tobacco leaves infected by TMV [118]. Both enzymes are able to hydrolyze so-called PR (pathogenesis related) plant proteins, which play an important role in protection from phytopathogenic microorganisms and are characterized by high resistance to conventional proteinases [102, 118]. Co-localization of proteinases and PR proteins in the extracellular space suggests that the enzymes play an important role in maintaining the necessary level of PR proteins, at least in plants of the Solanaceae family [118]. Increase in activity of aspartic proteinase was observed in intercellular fluid of potato tubers infected with *P. infestans*. The increase in activity was more pronounced in a disease-resistant variety [119]. Purified aspartic proteinase suppressed cyst germination in *P. infestans* and conidium germination in phytopathogenic fungus *Fusarium solani* [103]. The gene of the aspartic proteinase was found in *Arabidopsis*; hyperexpression of this gene increases the resistance to bacterial pathogens [120]. The gene product, proteinase CDR1, is accumulated in the intercellular fluid upon infection with bacteria with *Ps. syringae*. The effect of the proteinase results in the release of an elicitor of peptide nature [120]. The release of a signal peptide is blocked in the presence of aspartic proteinase inhibitor, pepstatin, and also as a result of mutations in the enzyme active site [121].

Secreted cysteine proteinase Rcr3 with properties similar to papain (C1A subfamily) was recently found in tomato leaves. The enzyme is involved in interactions between Avr2 (a protein from specific fungal tomato pathogen *Cladosporium fulvum*) and the product of a resistance gene, Cf-2 protein [104]. Different assumptions were made regarding the mechanism of proteinase action towards proteins [8]. It was recently established that Avr2 proteins acts as Rcr3 proteinase inhibitor, forming stable complexes with the enzyme. The interaction of these complexes with Cf-2 proteins induces a hypersensitive response in tomato plants [122].

Extracellular metalloproteinase genes have been identified in soybean (*Glycine max* (L.) Merr) and *Arabidopsis*. By their structure and properties, the proteinases SMEP1 from soybean and AtMMP from *Arabidopsis* are similar to metalloproteinases from animal matrix and belong to the same family of proteinases, M10A [105, 123]. Expression of soybean metalloproteinases was increased upon plant infection with pathogenic microorganisms *Ps. syringae* pv. *glycinea* and *Phytophthora sojae* [124]. The true physiological substrates for plant proteinases currently remain unknown. However, metalloproteinase Cs1-MMP from cucumber (*Cucumis sativa* L.) with similar properties is able to cleave proteins of the extracellular matrix in animals [125]. It has been assumed that plant metalloproteinases (similar to animal matrix metalloproteinases) can modify the extracellular matrix proteins resulting in the release of compound with antibacterial action [123, 124].

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