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REVIEW ARTICLE

Plant defense responses against viral and bacterial pathogen infections. Focus on RNA-binding proteins (RBPs)

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S u m m a r y

Plants have developed intricate defense mechanisms against pathogen infections. Immune system of medicinal plants is well developed. The molecular mechanisms of their ability to protect themselves are not fully understood. Little is known about RNA-binding proteins (RBPs) present in medicinal plants. However, CmGRP1 is an RBP found in the milky sap of medicinal plant *Chelidonium majus* L. what implies possible importance of RBPs in plant immunity. In this review recent insights into the role of plant RBPs in antiviral and antibacterial defense responses are discussed.

Key words: *plant defense, RNA-binding proteins (RBPs), glycine-rich proteins (GRP), pathogenesis-related proteins (PR), antimicrobial peptides (AMPs), antiviral response, Chelidonium majus* L.

INTRODUCTION

In evolution process, plants have developed intricate defense mechanisms against pathogen infections. The term “plant immune system” was coined by

Jonathan Jones and Jeffery Dangl in 2006 and it relates to the elaborate defense system against phytopathogenic microbes [1]. Its advancement and efficiency to suppress pathogen infections differ between plant species. Immune system of medicinal plants is possibly well developed what is indicated by their ability to protect themselves against various pathogens. However, the molecular mechanisms by which this immunity is achieved are not fully understood. Especially, little is known about RNA-binding proteins (RBPs) present in medicinal plants mainly because of the insufficient number of plant RBPs identified, however, CmGRP1 is an RBP found in the milky sap of medicinal plant *Chelidonium majus* L., what implies possible importance of RBPs in plant immunity [2, 3].

Generally, plant defense starts when a particular pathogen molecule or its structural feature is recognized by transmembrane protein recognition receptors (PRRs) on plant cell surface. The recognition is based on conserved features of molecules of bacterial or fungal origin, namely pathogen-associated or microbial-associated molecular patterns (PAMPs or MAMPs). This induces PAMP-triggered immunity (PTI) and the expression of defense genes, what prevents pathogenesis. However, some pathogens may release effector molecules and surpass PTI what leads to effector-triggered susceptibility (ETS). Subsequently, plants possess resistance (R) proteins usually containing nucleotide-binding (NB) and leucine-rich repeat (LRR) domains which trigger signaling cascade by recognizing specific effectors. This leads to the activation of downstream genes in order to create a robust and fast defense response preventing the spread of pathogens. The recognition of effector molecules by R proteins and the triggered defense response are known as effector-triggered immunity (ETI) (fig. 1). The signals from effector recognition are transmitted to the nucleus where they promote defense gene expression. These may code for transcription factors to commence the transcription of downstream enzymes required for the production of defense-related metabolites such as salicylic acid (SA) or pathogenesis-related (PR) proteins. Such signal transduction pathways lead to hypersensitive response (HR) characterized by accumulation of SA, reactive oxygen species, and the synthesis of PR proteins. The HR results in programmed cell death to stop pathogen invasion [4]. Regarding RNA viruses, they enter the plant cell via damaged tissues, what is typically the result of insect chewing or wounding. Then they remove the protein capsid and disassemble to release genetic material into the cytoplasm. Naked RNA becomes then a target of intricate defense mechanisms aiming to degrade infectious material, which are still not fully understood (fig. 1) [5].

Even though the aforementioned gene expression products have focused the greatest attention of the researchers, many genes encoding RNA-binding proteins (RBPs) have been recently found. RBPs allow plants to act also on a posttranscriptional level upon pathogen infection [6-9]. In its life, mRNA associates with RBPs which are responsible for processing, export from the nucleus, translation, and lifetime of pre-mRNA [10]. It was shown in *Arabidopsis*, that RBPs are involved in abiotic stress development, circadian rhythms, and flowering time [11, 12]. Finally, RBPs have been implicated in defense against pathogens [13].

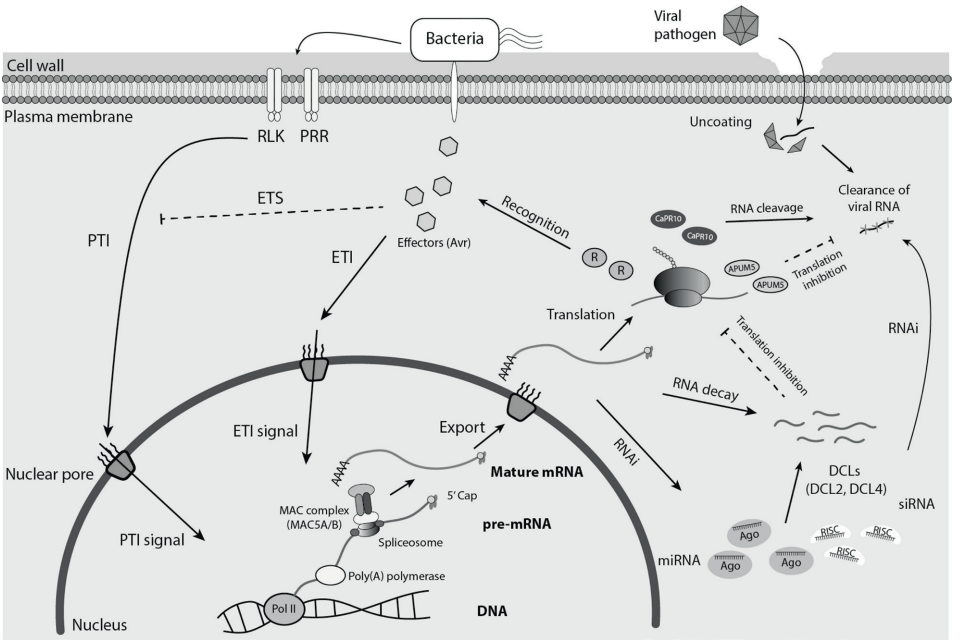

Figure 1.

Steps of RNA processing, regulating plant immunity. Signaling cascades are triggered by the recognition of pathogen associated molecular pattern (PAMP) by pathogen recognition receptors (PRRs), like receptor – like kinase (RLK), what induces PAMP triggered immunity (PTI). Bacterial pathogens can produce effector molecules that inhibit PTI what leads to effector-triggered susceptibility (ETS). Plants possess resistance (R) proteins containing leucine-rich repeat (LRR) and nucleotide binding (NB) motifs, which recognize the effectors and trigger effector-triggered immunity (ETI). Both PTI and ETI induce downstream defense genes followed by RNA processing steps such as 3' polyadenylation, splicing, 5' capping, and mRNA export out of the nucleus. MAC5A/B bind to the MOS4-associated Complex (MAC), which binds to the spliceosome, what suggests the contribution of MAC5A/B to mRNA splicing during plant defense against pathogens [6]. In case of viral pathogens after entering the plant cell via cell wall, disrupted by insect damage or wounding, they uncoat and disassemble. The viral RNA becomes a target for various protein- and RNA-mediated resistance mechanisms which function to silence infectious RNA. PR10 proteins including CaPR10, exhibit RNase activity and can cleave viral RNA [5]. Some RBPs such as APUM5 bind directly to viral RNA and repress its translation, what affects viral replication, movement, and symptoms [5]. DCL2 and DCL4 are involved in the formation of siRNAs in RNA interference (RNAi), which is a component of plant immunity [8, 27]. Being RNA-binding components of the RISC, AGO1, AGO2, and AGO7 participate in RNAi as well [27, 28]. Modified from [4].

One of recently identified plant RBPs is CmGRP1 isolated from medicinal plant *Chelidonium majus* L. [3]. The plant itself, also known as Greater Celandine is a perennial plant belonging to the family *Papaveraceae* and is broadly distributed in Southern and Central Europe, Western Asia, North America, and in the Azores archipelago [14-16]. It is a rich source of different biologically active substances and

it has been used in traditional medicine for many centuries [17-19]. In traditional European and Chinese herbal medicine, *Chelidonium majus* has been used to treat many diseases [16]. Its extracts were used against papillae, warts, and condylomas, which are a visible signs of human papillomavirus (HPV) infection. They were also exploited to treat liver disorders and fight fever. *Chelidonium majus* preparations have been frequently prescribed to treat gastric and biliary disorders, and irritable bowel syndrome [20]. In traditional Chinese medicine the plant is used as an antitussive, to treat whooping cough and bronchitis. Greater Celandine is also attributed to have antiviral, anti-microbial, and anti-inflammatory properties [21-23]. Alkaloids of *C. majus* are implied to be responsible for the aforementioned activities and are the major reason of the medicinal and pharmaceutical interest in this plant [24]. However, little is known about biological activities of *C. majus* proteins and their role in pathogenesis-related plant defense.

In this review recent insights into the role of plant RBPs in antiviral and antibacterial defense responses are discussed. Aspects of plant immunity involving RNA interference, HR, and others have been recently reviewed [25, 26], thus, they will not be thoroughly investigated in this paper.

CHARACTERISTICS OF RNA-BINDING PROTEINS (RBPs)

Structure and classification

In general, RBPs are various heterogenic proteins which contribute to different aspects of post-transcriptional regulation by direct interaction with RNA molecules – single or double stranded. These interactions are crucial for mRNA maturation events, such as splicing, 5' capping, polyadenylation, and export from the nucleus. Moreover, RBPs are further involved in post-transcriptional regulation in the cytoplasm including mRNA localization, mRNA stability, decay, and translation [29, 30]. Most RBPs are a part of versatile modular structures, with multiple repeats of few conserved domains, arranged in a variety of ways to fulfill their diverse functional requirements (fig. 2) [31].

RBPs contain one or more RNA-binding domains (RBD). The most popular domains in RBPs present in plants are RNA Recognition Motifs (RRM, also known as RBD or RNP domain) and K Homology (KH) domain [32]. These two types of domains have been also found in a broad spectrum of other species ranging from bacteria to humans, what suggests that they might be ancient protein structures [29]. Other common domains found in RBPs are zinc finger domain (ZnF, mostly C-x8-C-x5-C-x3-H type) [32, 33], cold-shock domain, DEAD/DEAH box [34], Pumilio/FBF (PUF) domain, double-stranded RNA binding domain (RS-RBD) [35], and Piwi/Argonaute/Zwille (PAZ) domain [36]. A single RBP may possess multiple binding domains [33-35]. Moreover, auxiliary motifs, such as arginine-rich, glycine-rich, arginine-glycine (RGG) or serine-arginine (SR) repeats are often present in

RBPs (fig. 2) [37]. Furthermore, it has been shown that RNA recognition motifs (RRMs) are responsible for the formation of heterogeneous ribonucleoprotein (RNP) complexes, by the involvement in RNA recognition and in protein-protein interactions [38]. Due to intricate functions and RBP modular structures, it is not easy to develop databases which can classify plant RBPs basing exclusively on RBDs. However, for experimentally identified RBPs in *Oryza sativa* a database has been established [39].

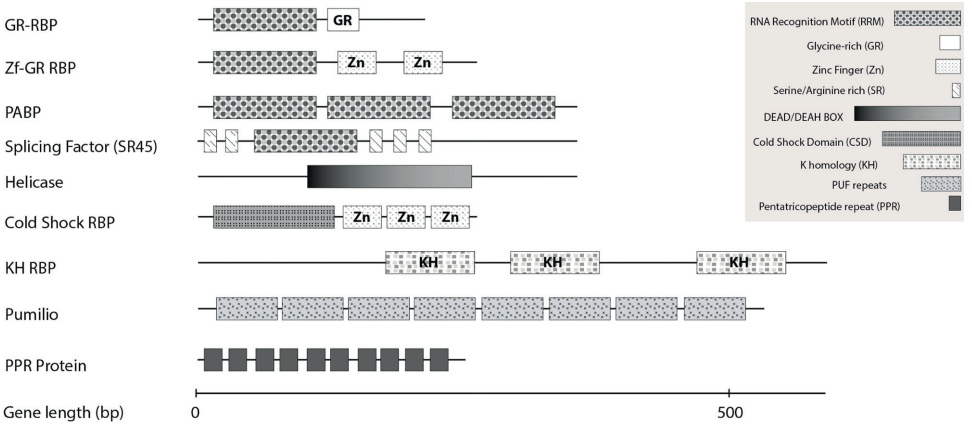

Figure 2.

Depicted modular structures from RNA-binding protein classes identified for *Arabidopsis thaliana*. In the box, RNA-binding domains and auxiliary motifs which are most common in plants. Modified from [48].

RBPs from plants are also present in cellular structures such as mitochondria, cytoskeleton, and telomeres [40]. In chloroplasts, they participate in RNA editing, intron splicing, and 3' end processing of chloroplast transcripts [38].

It is known that apart from activating transcriptional alterations as a defense reaction to a pathogen, plants also use post-transcriptional regulation of immune-responsive mRNAs. These reactions end up shaping the defense-related transcriptome which helps in plant defense against bacteria and viruses [41].

Plant glycine-rich proteins containing RBP domains

The family of proteins containing RNA-binding motifs is glycine-rich proteins (GRPs). They comprise of a large family of simple-structured, heterogenous proteins that consist of glycine-rich domains arranged in (Gly-X)n repeats, occupying from 20% to 70% of total amino acid residues of the protein [42, 43]. They are often included as a part of antimicrobial peptides (AMPs) protein family [44, 45]. GRPs are suggested to play an important role in RNA binding, protein-protein

interactions, and nucleolar targeting [3]. The presence of additional motifs as well as the arrangement of glycine repeats, groups them into four major classes [46], where the class IV can be further subdivided [3].

CmGRP1 is a newly described glycine-rich RNA-binding protein found in *C. majus* milky sap. It can be classified as a class IVa plant GRP. It means that apart from the glycine-rich domain, it has one RNA recognition motif (RRM), and it is implicated to be involved in plant defense [3]. The protein is composed of 146 amino acids and encoded by a 439 bp nucleotide sequence. The novel polypeptide was designated as CmGRP1 (*Chelidonium majus* glycine-rich protein 1) and its calculated molecular weight (MW) is 14.931 kDa. The CmGRP1 possesses a single conserved domain, the RNA recognition motif (RRM), which can be found in a variety of RNA-binding proteins (fig. 2). The domain contains four strands and two helices arranged in an α/β sandwich. During binding to RNA, in some cases a third helix is present as well. The C-terminal region of the protein is a disordered glycine-rich motif (GGGGxxGxGGGxxG). Using homology modeling approach, a 3D model of CmGRP1 structure was proposed [3]. Additionally, study using Real-Time PCR showed increased CmGRP1 mRNA levels observed in samples from plants subjected to viral pathogen infection as well as high salt conditions. It might suggest the involvement of this protein in plant responses against different stress conditions [47].

ROLE OF RNA BINDING PROTEINS IN PLANT IMMUNITY

The high degree of regulation of plant immunity occurs both transcriptionally and posttranscriptionally. Once transcribed, target gene RNA must be processed prior to translation. This includes polyadenylation, 5' capping, editing, splicing, and mRNA export. RBPs have been implicated at each level of RNA processing [4]. Cellular RNA-binding proteins perform diverse roles in cells, such as regulating RNA stability, splicing or mRNA export of immune-response transcripts [41]. Apart from the aforementioned cellular functions, RBPs are also a class of proteins which help plants to respond rapidly to environmental changes by being involved in a wide range of post-transcriptional regulatory events [48].

There is little information about plant RBPs as compared to those of other kingdoms, mainly due to the lack of proper plant-derived *in vitro* systems which would allow studying post-transcriptional events. However, more than 200 putative RBP genes have been predicted in the *Arabidopsis thaliana* genome [12], while approximately 250 RBP genes have been found in *Oryza sativa* [39]. Many of identified RBPs seem to be unique to plants, suggesting that they might serve plant specific functions, but still not many plant RBPs have been fully characterized [49].

As it was described above, there has been a limited number of RBPs identified in plants. Only some of them have been assessed for their role in plant immunity. A few RBPs not only consist of RNA binding motifs and consequently have putative functions in RNA processing. They are also suggested to play a role in plant

immune responses against microbes. Even though more research is required in this field, several examples of RBPs involved in plant defense against microbial pathogens are presented here.

Plant RBPs as susceptibility factors during viral pathogen infections

Eukaryotic initiation factor 4E (eIF4E) is thought to be a susceptibility factor against viruses [50, 51]. It comprises of an RBP that binds to the 5' cap-binding complex (CBC), which contributes to splicing and mRNA export out of the nucleus by associating with 5' mRNA immediately after its formation. eIF4E helps in the recruitment of other translation initiation factors by removing the secondary structure of 5' untranslated region (UTR) [52]. The eIF4E associates with the genome-linked proteins (VPg) of different potyviruses. In order to replicate potyviruses exploit host proteins such as eIF4E. To examine the susceptibility of *Arabidopsis* mutants *Ateif(iso)4e* consisting a transposon insertion were subjected to the infection of *Turnip Mosaic Virus (TuMV)*, *Lettuce Mosaic Virus (LMV)*, *Tomato Black Ring Virus (TBRV)*, and *Cucumber Mosaic Virus* strain R *(CMV R)* [51]. In response to *TuMV* infection *Ateif(iso)4e* mutant plants did not exhibit viral symptoms, while wild-type specimen were susceptible. Similar results were obtained during *LMV*-Most infection. This implies that these two potyviruses were not able to replicate in *Ateif(iso)4e* mutants and that the *AteIF(iso)4e* is required for their replication [51]. It was also shown that *AteIF(iso)4e* may be specifically required for replication of certain potyviruses, because during *TBRV* and *CMV-R* infection of *Ateif(iso)4e* mutants, similar viral symptoms were observed both in mutant and wild-type plants [51].

Plant RBPs involved in RNA interference

Several plant RBPs are involved in RNA interference (RNAi), a mechanism in which genes are being silenced after transcription [53, 54]. There are four Dicer-like proteins (DCL) found in *Arabidopsis*: DCL1, DCL2, DCL3, and DCL4 [55]. Two of them - DCL2 and DCL4 have been implicated to contribute to plant antiviral and antifungal response [8, 27]. DCL2 is involved in siRNA formation for viral RNA silencing [8], while DCL4 participates in sense transgene-induced RNA-silencing and is involved in siRNA production for endogenous gene regulation [56]. In one study *Arabidopsis* T-DNA insertional mutants *dcl2* and *dcl4* were subjected to a viral infection with *Tobacco rattle virus* (TRV-PDS), which does not affect wild-type *Arabidopsis* plants. There was no change in siRNA accumulation in *dcl2* mutants. However, in *dcl4* mutants, the typical 21-nucleotide siRNAs which are normally found during viral infection, were replaced by 22-nucleotide siRNAs [8]. Still, no viral RNAs are accumulated in single mutants what suggests that they can be functionally redundant. In order to test this, the two single mutants were crossed, creating *dcl2 dcl4*

double mutants, which accumulated 24-nucleotide siRNAs, displayed viral RNA accumulation, and showed viral disease symptoms. The study shows that siRNAs produced by DCL2 and DCL4 contribute to RNA silencing of *Tobacco rattle virus* [8]. This demonstrates the role of RBPs in plant defense against viral pathogens via RNAi processes.

Plant RBPs involved in defense responses against bacterial pathogens

MAC5A and MAC5B are RNA-binding proteins that bind to MOS4-associated complex (MAC), which is involved in plant immunity and mRNA splicing [57, 58]. They are highly homologous one to other and both have an RRM as well as a CCCH-type zinc finger domain [6]. In order to assess the contribution of these proteins in plant immunity, the T-DNA insertional *snc1 mac5a* and *snc1 mac5b* double mutants were used. The *snc1* single mutant background exhibits defense responses and increased resistance against pathogens [59]. The *snc1 mac5a* double mutant was more susceptible to infection than *snc1* by the bacterial pathogen *Pseudomonas syringe* pv. *tomato* (*P.s.t.* DC3000). However, lack of susceptibility to the pathogen in case of *snc1 mac5b* mutants was observed. This resistance, similar to the one in *snc1* single mutants may be due to the fact that *snc1* phenotype dominates over the *mac5b*, while *mac5a* suppresses *snc1* resistance. This agrees with the unequal redundancy of these two genes and suggests that MAC5A/5B are involved in plant immune responses against bacterial infections.

RBPs AGAINST VIRAL RNA INFECTION: TWO WAYS OF ACTION

Plant RBPs are involved in immune response via direct and indirect interactions with viral RNA [5]. Hereafter several examples are described.

Plant RBPs regulate host RNA or degrade viral RNA during viral pathogen infection

RBPs can function in sequence-specific or non-specific manner. In many cases RBPs associate with host RNA at mRNA levels and regulate defense signaling pathways during RBP-mediated defense response to pathogens.

Arabidopsis thaliana RNA-binding protein-defense related 1 (AtRBP-DR1) is an example of RBP that acts by binding to target host RNAs, what positively contributes to hemibiotrophic pathogen defense and possibly governs genes engaged in the salicylic acid (SA) signaling pathway in the cytosol [60].

Glycine-rich RNA-binding protein 7 is involved in plant immunity against such pathogens as *Pst* DC3000, necrotrophic bacterium *Pectobacterium carotovorum*

SCC1 as well as the biotrophic virus *Tobacco mosaic virus* [61]. Data from global transcript profiling between the wild type and AtGRP7-overexpressing transgenic plants were compared. The study showed that AtGRP7 governed approximately 300 transcripts including those involved in stress response, circadian clock, ribosome function, and RNA metabolism [62]. Still, the RNAs targeted by these RBPs and their RNA-binding domains were not identified. Little is known about the RBPs-mediated defense and its mechanism of action. It was suggested that RBPs generally control defense signaling-related genes during pathogen infections at posttranscriptional/translational levels [5].

As it was mentioned above, some RBPs govern host innate immunity during pathogen infections and some are directly involved in virus resistance via binding to viral RNAs.

CaPR10 is an RBP isolated from hot pepper (*Capsicum annum*) belonging to the pathogenesis-related protein family 10 (PR10). The PR10 proteins exhibit ribonuclease-like properties and contain highly conserved regions such as a specific domain (KAXEXYL), and the P-loop domain (GXGGXGXXK), which is an RNA-binding site. However, the specificity of binding affinity of these regions to target RNA remains to be solved. Upon viral infection, CaPR10 is directly involved in plant defense and its enhanced ribonucleolytic activity to viral RNAs is well known [63].

Although PR10 proteins act as RBPs, it is still not clear if they specifically contribute to RNA virus defense, as this protein family is also known to participate in defense responses upon different abiotic and biotic stresses [64, 65]. Even though the actions of the PR10 family seem to be non-specific, it was suggested that proteins belonging to this family could use helper proteins for specific binding of target RNAs, including viral or host RNAs [5].

PLANT RBPs REPRESS VIRAL RNA DURING TRANSLATION

Subjected to virus infection, plants are capable of recognizing the invading RNA virus and triggering innate immune system to suppress viral infection. However, little is known about mechanisms which detect RNA of infecting viruses. As a part of the viral RNA defense system, RBPs directly associate with the viral RNA and effectively affect replication and movement of the plant RNA virus. Several RBPs are known to bind to the viral RNA directly.

Pumilio-fem-3 mRNA binding factor (PUF) is an RBP which is known as a post-transcriptional/translational repressor in mammalian systems. It binds to the 3' UTRs of its target mRNAs and contains a highly conserved Pumilio homology domain (PHD) at the C-terminus [66]. It has been suggested that eight α-helical repeats of PHD are responsible for the recognition and binding affinity of target RNA [67].

Arabidopsis Pumilio RNA-binding protein 5 (APUM5) has a conserved PHD and it directly associates with the "UGUA"-containing nucleotides in the 3' UTR, as well as with internal regions of *Cucumber mosaic virus* (CMV), suppressing its replication.

Moreover, APUM5 also suppresses translation [5]. Putative PHD-binding core motifs can be also found in *Turnip mosaic virus* (TuMV) genome. In one study, *APUM5*-overexpressing transgenic plants were subjected to TuMV infection. At the early stage, the transgenic plants presented decreased TuMV RNA levels and slightly enhanced resistance compared to wild-type plant [68]. APUM5 is implied in viral RNA-targeted plant defense. It may also control unknown host target RNAs in RNA sequence-specific way [5].

CONCLUSIONS

As described above, relatively small number of RBPs has been characterized. Due to the similarity of RBPs present in plants and metazoa, studying plant RBPs in response to pathogen infection may contribute to our understanding of similar RBPs in other kingdoms [32]. CmGRP1 identified in *C. majus* is an example of many potential medicinal plant RBPs which activity during viral pathogen infection is yet to be solved. However, because of the general instability of their mRNA targets, and the lack of appropriate plant-derived *in vitro* systems, RBPs are difficult to study. Next generation sequencing techniques will greatly improve our ability to analyze RBPs and knockout mutants of RBPs. The knowledge about the mechanism of the effective virus RNA suppression via RNA-binding proteins can also be potentially exploited in crop engineering as a synthetic virus defense strategy.

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ODPOWIEDŹ OBRONNA ROŚLIN PRZED ZAKAŻENIEM WIRUSOWYM I BAKTERYJNYM – ROLA BIAŁEK WIĄŻĄCYCH RNA (RBPs)

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Streszczenie

Rośliny wykształciły szereg mechanizmów obronnych przed zakażeniem patogenem. System obronny roślin leczniczych jest dobrze rozwinięty, na co wskazuje ich odpowiedź przeciw różnym infekcjom. Jednak mechanizmy molekularne tych odpowiedzi nie są do końca poznane. Jak dotąd niewiele wiadomo na temat białek wiążących RNA (RBPs) z roślin leczniczych. Jednym z takich białek jest CmGRP1 zidentyfikowane w soku mlecznym glistnika jaskółcze ziele (*Chelidonium majus* L.), co wskazuje na potencjalnie duże znaczenie tej grupy białek w obronie roślin. W niniejszym artykule przeglądowym przedstawione są najnowsze informacje dotyczące roślinnych białek RBP i omówione jest ich znaczenie dla obrony roślin przeciw patogenom wirusowym i bakteryjnym.

Słowa kluczowe: *obrona rośliny, białka wiążące RNA (RBPs), białka bogate w glicynę (GRP), białka związane z patogenezą (PR), peptydy przeciwdrobnoustrojowe (AMPs), odpowiedź przeciwwirusowa, Chelidonium majus L.*