Minireview

Antibiotic activities of peptides, hydrogen peroxide and peroxynitrite in plant defence

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Abstract Genes encoding plant antibiotic peptides show expression patterns that are consistent with a defence role. Transgenic over-expression of defence peptide genes is potentially useful to engineer resistance of plants to relevant pathogens. Pathogen mutants that are sensitive to plant peptides in vitro have been obtained and a decrease of their virulence in planta has been observed, which is consistent with their hypothetical defence role. A similar approach has been followed to elucidate the potential direct anti-microbial role of hydrogen peroxide. Additionally, a scavenger of peroxynitrite has been used to investigate its involvement in plant defence. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Innate immunity; Active oxygen; Nitrogen species; Plant disease resistance; Cell death; Urate; Plant–microbe interaction

1. Introduction

Plants have complex defence mechanisms that are either pre-formed or activated in response to pathogen attack [1,2]. Resistance or susceptibility to a particular pathogen depends on various factors, including pathogen recognition, activation of signal transduction pathways and elicitation of active and passive defence molecules [2]. In vitro anti-microbial activity has been demonstrated for the following types of molecules: (i) some of the so-called pathogenesis-related proteins, which were originally identified as pathogen-elicited proteins of unknown function [2]; (ii) a considerable variety of plant organic compounds, classified into phytoanticipins and phytoalexins, which include phenols and phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenetic glycosides, glucosinolates, 5-alkylated resorcinols and dienes [3]; (iii) a number of plant anti-microbial peptide families [4]; and (iv) active oxygen and nitrogen species, such as hydrogen peroxide and peroxynitrite [5]. Demonstration of a possible in vivo defence role for a given antibiotic agent involves observations of diverse nature, which include the finding of a correlation between expression levels and the severity of symptoms and/or between pathogen resistance to the agent and virulence. This review will focus on recent evidence concerning the possible in vivo antibiotic activities of peptides, hydrogen peroxide and peroxynitrite, as well as on the pathogen response to these challenges (Fig. 1).

2. Novel families of plant anti-microbial peptides

A number of antibiotic peptide families have been described in plants (Table 1). Until recently, only globular peptides, stabilised by disulphide bonds, had been identified in plants [4,6]. Thionins were the first whose activity against plant pathogens was demonstrated in vitro [7]. Subsequently, several families of cysteine-rich peptides have been characterised, including defensins [4], lipid transfer proteins (LTPs) [4,6], hevein-type peptides [4] and knottin-type peptides [4], as well as peptide MBP-1 from maize [8] and a group of 20-residue peptides (Ib-AMPs) isolated from the seeds of *Impatiens balsamina* [9,10]. Novel plant antibiotic peptides include the following types: the snakin/GASA family of 12-cysteine peptides ([11], Berrocal-Lobo et al., unpublished), which have been first isolated from potato and are ubiquitous, as judged from the multiplicity of homologous cDNAs that have been reported [12,13]; the shepherdins, which are linear glycine/histidine-rich peptides isolated from the roots of shepherd's purse (*Capsella bursa-pastoris*) [14]; and the macrocyclic cysteine-knot peptides that have been purified from different plants of the Rubiaceae family (coffee and other tropical plants) in a screening for anti-HIV compounds [15].

3. Expression of peptide-encoding genes and disease tolerance

Correlation of altered peptide levels in planta with variation of disease tolerance is indicative of the possible defence role of the peptides [16–21]. Observations concerning thionins, defensins and LTPs are consistent with the defence hypothesis. Thionin mRNA is transiently induced in barley upon infection with *Erysiphe graminis* in both susceptible and resistant cultivars [22,23]. Transgenic tobacco plants expressing a barley thionin gene showed reduced lesion size when the plants were challenged with two strains of *Pseudomonas syringae* [23], whereas other strains did not seem to be affected [24]. Subsequently, over-expression of an endogenous thionin was

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Abbreviations: LPS, lipopolysaccharide; LTP, lipid transfer protein; AOS, active oxygen species

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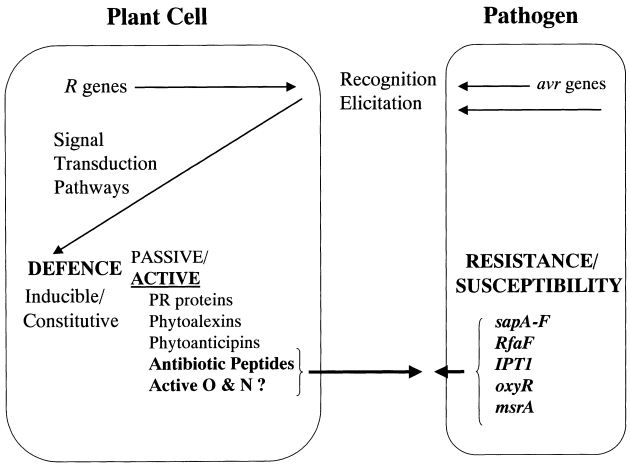

Fig. 1. Simplified scheme of plant–pathogen interactions. Aspects discussed in this review are highlighted in bold letters (see text for explanations).

reported to enhance resistance of *Arabidopsis thaliana* against *Fusarium oxysporum* [17] and *Plasmodiophora brassicae* [20].

Experiments with radish seeds have demonstrated that defensins represent over 30% of the proteins released during germination (about 1 μg/seed) and that the released defensin is sufficient for fungal inhibition, an effect that may contribute to the enhancement of seedling survival rate [18]. Defensin *PDF1.2* gene from *Arabidopsis* is upregulated by jasmonate and ethylene, as well as by infection with the fungal pathogens *Alternaria alternata* and *Botrytis cinerea* [25]. Mutants impaired in the jasmonate and ethylene signal transduction pathways, which do not express gene *PDF1.2*, show enhanced susceptibility to these necrotrophic fungal pathogens [26,27]. A more direct evidence of an in vivo role for defensins is provided by over-expression experiments. Thus, transgenic expression in tobacco of the Rs-AFP2 defensin from radish (up to 0.2% of leaf proteins) resulted in a seven-fold reduction in lesion size with respect to the non-transformed control, upon infection with the foliar fungal pathogen *Alternaria longipes* [18], and over-expression of an anti-fungal defensin from *Medicago sativa* in potato conferred robust resistance under field conditions [28].

It has been shown that *LTP* genes respond to pathogen infection in a complex manner, as they can be induced above basal levels or be switched off by different plant pathogens that infect barley [6,29,30]. Thus, infection by the fungal pathogen *Rhynchosporium secalis* increases *LTP* gene expression only in the incompatible interaction, not in the compatible one, and this induction is under the control of a resistance gene (*Rh3*); and infection with the compatible bacterial pathogen *P. syringae* pv. *japonica* switches off *LTP* gene expression [6,30]. Also, induction of *LTP* genes by cauliflower mosaic virus infection in *Arabidopsis* [31], by *Xanthomonas* sp. in pepper [32] and by arbuscular mycorrhiza in rice [33] have been reported. The possible defence role of LTPs is further supported by the observation that transgenic tobacco and *Arabidopsis* plants over-expressing a barley LTP showed drastic reduction of disease symptoms after infection of the leaves with the bacterial pathogen *P. syringae* [21].

4. Pathogen sensitivity to plant peptides and virulence

In agreement with the defence hypothesis, peptide-sensitive mutants of the pathogens show significantly decreased virulence towards plant tissues in which these peptides are present [34,35]. Furthermore, the latter type of evidence indicates that both plant and animal pathogens deal in a similar way with host defences, as the equivalent mutants of animal pathogens show also decreased virulence [35,36]. The possibility that the pathogen defence system against anti-microbial peptides may show specificity towards the peptide type has been suggested and might be highly relevant in plant–pathogen interactions [35].

A first type of mutant with increased sensitivity to thionins and LTPs was obtained by insertion of transposon Tn*5* in *Ralstonia solanacearum*. This mutation interrupted the *rfaF* gene, which encodes a heptosyl transferase involved in the synthesis of lipopolysaccharide (LPS). Consequently, LPS of the mutant lacked heptose and the phosphate groups that reside in this sugar, and the mutant was avirulent in planta [34].

It seems that phosphate groups in the LPS act as traps for the peptides and prevent their interaction with target sites [34]. Also, a defensin-resistant mutation in gene *IPT1* of *Saccharomyces cerevisiae*, which prevents phosphorylation of a membrane sphingolipid, mannose-(inositol-phosphate)2-ceramide, has been recently reported [37].

A second type of peptide-sensitive mutants are affected in the *sap* operon (for sensitive to anti-microbial peptides), which has been well studied in *Salmonella typhimurium*, where it is required for peptide resistance and for virulence in mice [38]. The *sap* genes are organised in a single operon and exhibit sequence similarity with ABC transporters described in prokaryotes and eukaryotes. The proposed mechanism of action for the Sap system includes binding of the periplasmic component SapA to the anti-microbial peptide, followed by peptide transport to the cytoplasm, where peptide degradation and/or activation of resistance determinants occur. The *sapA*

Table 1
Plant anti-microbial peptides

Peptide family	Number of residues	Disulphide bridges	Types/subfamilies	Active against
LTPs	90–95	3–4	I–II	bacteria and fungi
Snakins (GASA)	61–70	6	I–III	bacteria and fungi
Defensins	45–54	4	I–IV	bacteria and fungi
Thionins	45–47	3–4	I–IV	bacteria and fungi
Hevein-like	43	4	I	Gram(+) bacteria and fungi
Knottin-like	36–37	3	I	Gram(+) bacteria and fungi
Shepherdins	28–38	0 (linear)	I–II	bacteria and fungi
MBP-1	33	2	I	bacteria and fungi
Macrocyclic peptides	29–31	3	I–III	Gram(+) bacteria
Ib-AMPs	20	2	I	Gram(+) bacteria and fungi

to *sapF* operon from the pathogenic bacterium *Erwinia chrysanthemi* has five open reading frames that are closely related (71% overall amino acid identity) and are in the same order as those of the *sapA* to *sapF* operon from *S. typhimurium*. An *E. chrysanthemi sap* mutant was more sensitive to wheat α-thionin and to snakin-1, and also less virulent than the wild-type strain in potato tubers. These results indicate that the interaction of anti-microbial peptides from the host with the *sapA* to *sapF* operon from the pathogen plays a similar role in animal and in plant bacterial pathogenesis [35] and, indeed, the *sap* operons from *Erwinia* and *Salmonella* showed reciprocal functional complementation (López-Solanilla et al., unpublished results). Moreover, the mutation in the *sap* locus had a greater effect on virulence than those in other well-characterised gene systems involved in plant–pathogen interactions [39], such as the PelABCE locus [40], which codes for pectate-lyases, and the Hrp locus [41], which codes for a type III secretion system.

5. Active oxygen and nitrogen species versus virulence

A similar approach to that followed with the antibiotic peptides has been used to ascertain the possible in vivo anti-microbial properties of active oxygen species (AOS), which may play a dual role in defence: a direct antibiotic activity and an indirect effect as mediators of the activation of other defence components [42]. Although the in vitro activity of O_2^- and H_2O_2 against phytopathogenic bacteria has been reported [43], its role in vivo remains controversial. Thus, a mutation of the *oxyR* gene, which controls several enzymes involved in AOS detoxification, had no effect on virulence of *E. chrysanthemi* [44]. OxyR is a transcriptional activator of genes encoding several enzymes, including catalase and glutathione reductase [45]. The *E. chrysanthemi oxyR* mutant strain, which was more sensitive to H_2O_2 and was unable to form individual colonies on solid medium unless catalase was added exogenously, retained full virulence in potato tubers and tobacco leaves. Moreover, both the wild-type strain and the *oxyR* mutant were insensitive to exogenously added H_2O_2 when inoculated into the plant. These data point towards a lack of direct anti-microbial effect of H_2O_2 in the plant defence against *Erwinia* invasion, possibly because the combined effects of anti-oxidant enzymes and reductant molecules from the plant prevent H_2O_2 from reaching concentrations that are lethal to the bacteria. In contrast, El Hassouni et al. [46] reported that the *msrA* mutant of *E. chrysanthemi*, which affects an enzyme that repairs oxidised proteins, was more sensitive to oxidative stress and had diminished virulence in *Chicorium intibus* (chicory) and *Saintpaulia ionantha*. The interpretation of these results is complicated by the pleiotropy of the *msrA* mutation, since the diminished virulence could be due to either increased sensitivity to oxidative stress, altered motility or other unknown effects of the mutation.

Nitric oxide (NO) has been recently demonstrated to play a prominent role during plant hypersensitive response and cell death [47–49]. One likely role for NO and AOS is to promote plant cell death and pathogen killing, as in the mammalian inflammatory response, probably by reaction of NO with O_2^- to produce peroxynitrite [50–53]. However, it is unclear whether NO or its activated derivatives are directly toxic to pathogens in plants. In vitro growth of both a virulent (*P. syringae pv. phaseolicola* 110; *avr*RPM1$^-$) and an avirulent (*P. syringae pv. phaseolicola* 411; *avr*RPM1$^+$) bacterial strain was inhibited by NO, as well as by the peroxynitrite generating system sodium nitroprusside+hypoxanthine/xanthine oxidase, and direct application of peroxynitrite induces plant cell death, which is prevented by the peroxynitrite scavenger urate [5]. Using urate, it has been shown that although peroxynitrite was responsible for most of the host cell death of *Arabidopsis* in response to the avirulent *P. syringae* strain (Fig. 2), scavenging of peroxynitrite did not compromise the effective defence against this avirulent pathogen, in spite of the reduction in plant cell death [5]. Although peroxynitrite has been suggested as being responsible for direct pathogen killing [52,53], urate scavenging of toxic peroxynitrite did not lead to a higher growth of either the virulent or the avirulent strains, which indicated that peroxynitrite toxicity was not limiting bacterial growth in planta [5]. On the contrary, the use of the urate promoted discrete death of plant tissue challenged with the virulent strain *P. syringae* pv. *phaseolicola* 110 (*avr*RPM1$^-$) (Fig. 2) and resulted in a severe growth restriction of the pathogen [5]. This complex situation may parallel

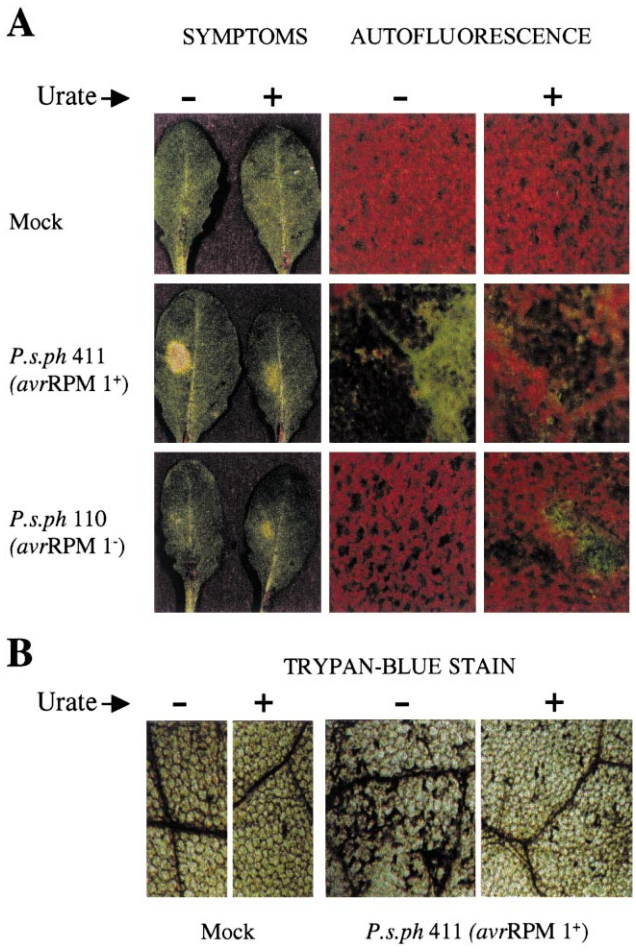

Fig. 2. Effects of urate, a scavenger of peroxynitrite, on the plant response to bacterial pathogens [5]. A: Col-0 *Arabidopsis* plants were inoculated with 10^6 cfu/ml of avirulent, *avr* RPM1$^+$, *P. syringae pv. phaseolicola* 411 (*P.s.ph* 411); or virulent, avrRPM1$^-$, *P. syringae pv. phaseolicola* 110 (*P.s.ph* 110), with or without 1 mg/ml uric acid. After 24 h, leaves were examined for visible symptoms (left) or ultraviolet-stimulated autofluorescence (right). B: Microscope photographs of control and *P.s.ph* 411 inoculated leaves, with and without urate stained with the Trypan-blue dye, at 24 h post infection.

that in animal systems where protective and toxic effects have been suggested for nitric oxide-related compounds [54–56].

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