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Bacterial lipopolysaccharides and plant–pathogen interactions

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Abstract

Lipopolysaccharides are amphipathic molecules forming the outermost layer of the cell surface of Gram-negative bacteria. They are essential for protecting the cell from hostile environments and, in the case of pathogens, they play a direct role in interactions with eukaryotic host cells. Mutants with altered lipopolysaccharide structure have been obtained with several plant pathogenic bacteria; such mutants generally show reduced virulence. Purified lipopolysaccharide has several effects on plants, notably suppression of the hypersensitive response to subsequently inoculated avirulent pathogens. The suppression is strictly localized and is observed within a time 'window' of, typically, 10–30 h. Although infiltration of lipopolysaccharide into leaves produces no macroscopic symptoms, characteristic changes in plant gene expression can be observed. One effect is to sensitize the plant tissue to subsequent bacterial inoculation so that the sensitized tissue responds more rapidly and intensely, giving partial inhibition of bacterial growth. The synthesis of antimicrobial hydroxycinnamoyl tyramine conjugates is one facet of the process which provides an excellent biochemical model for analysing the phenomenon. Lipopolysaccharide induces the synthesis of two enzymes involved in conjugate production (tyrosine decarboxylase and tyramine-hydroxycinnamoyl transferase), but the conjugates themselves are not produced until bacteria are subsequently inoculated. Using this and other examples we discuss the mechanisms of lipopolysaccharide action on plants in the context of plant disease.

Abbreviations: CT – coumaroyl tyramine; FT – feruloyl tyramine; HR – hypersensitive response; LIR – localised induced response; LPS – lipopolysaccharide; PAL – phenylalanine-ammonia lyase; THT – tyramine-hydroxycinnamoyl transferase; TyDC – tyrosine decarboxylase.

Introduction

The cell envelope of Gram-negative bacteria contains two bilayer membranes. The outer leaflet of the outer membrane contains lipopolysaccharide (LPS), a complex family of amphipathic molecules which play important roles in the interaction of bacteria with the extracellular environment, including eukaryotic hosts (Rietschel et al., 1994). LPS is a tripartite molecule. The outermost layer, exposed to the aqueous extracellular environment of the bacteria, is called the O antigen and consists of a chain of oligosaccharide repeat units. The O antigen is the immunodominant component of the cell surface and many serotype classifications of bacteria reflect variation in O antigen structure. The O antigen is not essential for bacterial viability and mutants lacking it can be readily isolated. Such mutants are called 'rough' because of the appearance of colonies, contrasting with the 'smooth', more mucoid appearance of wild type colonies. In the case of plant pathogens, O antigen may not be essential for pathogenicity. For example, some strains of *Xanthomonas campestris* isolated from naturally diseased plants lack O antigen. The second domain of LPS comprises the inner and outer oligosaccharide core. Mutants can be obtained lacking the outer

core, although such mutants are compromised in resistance to chemical and physical stress and have lost pathogenicity. The inner core contains the unusual sugar KDO (3-deoxy-D-manno-2-octulosonate) and is linked to the third domain, lipid A. Lipid A consists mainly of a glucosamine dimer N- and O-substituted with medium chain length hydroxyfatty acids. The fatty acid chains form the outer bilayer of the outer membrane. Our view of LPS structure is based mainly on studies with the enteric bacteria, *Escherichia coli* and *Salmonella* spp. Detailed structural studies have been carried out on few other bacterial groups, but partial chemical characterization suggests that the LPS of plant pathogens shares similar general features with enteric bacterial LPS.

The effects of LPS on plant defence responses have been comprehensively reviewed (Dow et al., 2000). This detailed survey will not be repeated here. This paper will give a general overview of the topic and discuss possible future research strategies. Although studies of LPS action on plants began thirty years ago, the literature is relatively sparse. Biochemical and physiological work in the 1960s and 1970s has been supplemented with genetic analysis in the 1990s, and the results of genomic approaches which are now commencing are awaited with interest. The lack of research on LPS is a consequence of the difficulties caused by the complexity of the chemical structure of LPS and the rather subtle nature of its effects on plants.

LPS is required for virulence

Mutants with defects in LPS biosynthesis have been described in all the major genera of bacterial pathogens (Dow et al., 1995; Drigues et al., 1985; Hendrick and Sequeira, 1984; Kao and Sequeira, 1991; Kingsley et al., 1993; Schoonejans et al., 1987; Titarenko et al., 1997). Such mutants usually give reduced symptom severity in plants and the number of viable bacteria in plant tissue declines rapidly. LPS-defective mutants are more sensitive to agents such as some antibiotics, detergents, and antimicrobial peptides in culture, probably because the defective LPS can no longer exclude these agents from the cell. It is possible that the killing of mutant bacteria in plant tissues may reflect an inability to exclude toxic compounds produced by plants. The hydroxycinnamoyl tyramine conjugates described below are a good example of such compounds.

In addition to its protective function for the bacteria, LPS may interact directly with hosts. This may in principle be mediated by direct contact of bacteria with host cell surfaces or as a consequence of release of LPS from the bacteria. It is not known whether direct contact is involved in LPS-mediated effects, but it has recently been shown that a network of functions required for virulence of *Ralstonia* (formerly *Pseudomonas*) *solanacearum* is activated by contact with plant cells (Aldon et al., 2000). Presumably, interactions leading to modulation of plant gene expression (discussed below) would require recognition of LPS by components within or attached to the plant cytoplasmic membrane. The plant cell wall probably prevents direct contact of the membrane with LPS of apposed bacteria. Moreover the outermost portion of the LPS molecule, the O antigen chain, is not the active moiety in triggering plant responses. In animal pathogenesis, bacteria can be closely associated with host cell membranes and direct contact of LPS with surface receptors is possible. However it should be noted that in animals the active moiety of LPS is lipid A, which is buried in the bacterial outer membrane. An alternative possibility to LPS perception by direct bacteria-cell contact is that detachment of LPS from the bacterial surface is necessary. A significant fraction of LPS may be released from bacteria as micelles or blebs during growth (Beveridge, 1999) and this may be the form in which LPS interacts with eukaryotic cells. Blebs may also sequester periplasmic contents and deliver the material to other cells by fusion with membranes. Indeed, it has been suggested that this process may represent a widespread alternative route for periplasmic protein secretion (Beveridge, 1999). The effects of LPS on animal cells have been studied extensively (reviewed by Medzhitov and Janeway, 1997, 1998; Qureshi et al., 1999). It is not known whether there are mechanistic parallels between animal and plant responses to LPS.

LPS can prevent induction of the hypersensitive response

Early observations showed that heat-killed cells of *R. solanacearum* infiltrated into tobacco leaves influenced the response to subsequent inoculation with living bacteria. The pretreatment could prevent or delay either the hypersensitive response (HR) induced by avirulent bacteria or disease symptoms induced by virulent pathogens. Fractionation of bacteria showed that LPS was responsible for the suppression. (LPS is not affected by the heat-killing process). The source of

LPS used was not critical: preparations from a number of bacteria, including non-pathogens, were effective (Mazzucchi and Pupillo, 1976; Mazzucchi et al., l979; Minardi, 1995). The phenomenon can be observed with many challenging bacteria and in many plant species, although solanaceous plants have been most commonly used. However, there are no reports concerning monocotyledons. In pepper (*Capsicum annuum*) cv ECW10R, which carries the *BsI* resistance gene against bacterial speck, HR can be induced in the gene-for-gene interaction with *Xanthomonas campestris* pv *vesicatoria* carrying the *avrBsI* avirulence gene or in the 'non-host' interaction with the related crucifer pathogen *X. campestris* pv *campestris*. Pretreatment with LPS preparations from enteric bacteria and from several *X. campestris* pathovars prevented HR in both cases (Newman et al., 2000).

The prevention of HR by LPS is strictly localized to the area of the leaf into which the LPS is infiltrated; adjacent areas of the leaf behave normally when subsequently challenged. The effect is independent of light. LPS treatment by itself does not cause necrosis or other visible symptoms. The prevention of HR requires several hours to become established, and is temporary. For example, in pepper the interval between LPS administration and challenge inoculation needs to be between 10 and 30 h to demonstrate HR suppression (Newman et al., 2000). In tobacco, there are reports that LPS can also induce a second phase of resistance that is light-dependent, long-lasting and systemic (Klement et al., 1999; Sequeira, 1983), but the relationship of this to the first, temporary and localized phase is not known. The concentration of LPS required to give the localized suppression of HR induction is around 50 $\mu g\ ml^{-1}$. However, it is difficult to interpret this value because of uncertainties about the physical state of LPS preparations. The predominant form in aqueous preparations is likely to be micellar, but it cannot be ruled out that the preparations contain small amounts of a more readily accessible form which is active.

Sequeira (1983) used the term 'localized induced resistance' (LIR) for the effect of LPS in preventing HR. We retain the abbreviation LIR, but we prefer the more general term 'localized induced response'. One reason for this is that HR is generally regarded as a manifestation of resistance of plants to pathogens. Prevention of HR cannot therefore logically be considered as induced resistance.

Under the conditions used to demonstrate HR and LIR, active growth of the challenging pathogen in the plant tissue is necessary. LIR is believed to operate by creating an antimicrobial environment within the plant (Rathmell and Sequeira, 1975), although the induction of increased tolerance of plant tissue to bacteria has also been suggested (Mazzucchi et al., 1979). A mutant of *X.c. campestris* with altered LPS structure was more sensitive to certain antibiotics and inhibitors (Dow et al., 1995) and showed transient inhibition of growth in a normal host plant, turnip. However, in LPS-pretreated pepper leaves the mutant was rapidly killed, consistent with the presence of an antimicrobial substance in the plant (Newman et al., 2000).

In incompatible plant–bacterial interactions the development of the HR is usually associated with a decline in the number of viable bacteria which can be recovered from the tissue. However, the magnitude and kinetics of the effect vary according to the experimental conditions, such as the concentration of bacteria in the challenge inoculum. Survival of bacteria was reported to be enhanced in LPS-pretreated leaves (Minardi, 1995; Newman et al., 2000; Sequeira and Hill, 1974), although no net increase in bacterial numbers was observed. In the incompatible interaction between pepper and *X.c. campestris* bacterial numbers declined over the first 24 h in both normal and LPS-pretreated tissue when a high inoculum density was used. Thereafter, numbers remained high in LPS-pretreated leaves, but continued to decline in untreated leaves. When a lower inoculum density was used, LPS pretreatment caused a transient reduction in growth during the first 24 h only (Newman et al., 2000). In incompatible interactions with *X.c. vesicatoria*, on the other hand, LPS pretreatment gave some inhibition at a low inoculum density, but no significant differences were seen at a higher inoculum density. HR induction by moderate to high inocula was associated with rapid local necrosis of plant tissue. LIR does not appear to work by inhibiting necrosis *per se* because elicitors such as harpin or Avr9 were still able to induce necrosis in LPS-pretreated tissue (Newman et al., 2000).

The structural requirements of LPS for prevention of HR have been studied by two methods: chemical degradation and the use of truncated LPS forms produced by mutant bacteria. Early work with the tobacco–*R. solanacearum* system implicated the lipid A-core region of LPS as the active moiety (Graham et al., 1977). In the pepper–*X. campestris* system, use of LPS from *S. minnesota* mutants showed that LPS from the Rd mutant, consisting of lipid A plus inner core, was active, whereas lipid A alone was inactive (Newman

et al., 1997). The O antigen and outer core regions were not necessary for activity. Using hydrolyzed LPS from *X. campestris*, similar conclusions were drawn: O antigen and lipid A were inactive, but the lipid A core complex retained the full activity of the native LPS. However, in contrast to *S. minnesota* LPS, the core alone of *X. campestris* LPS (lacking lipid A) was also active (Newman et al., 1997). In interpreting the apparent lack of activity of lipid A alone on plant cells it must be pointed out that free lipid A is insoluble in water and may be unable to reach the plant cell membrane. Conjugation to the core oligosaccharide may overcome this problem by maintaining lipid A in a form in which it can be presented to the plant membrane. Although it remains possible that lipid A has activity, the results with the *X. campestris* core oligosaccharide suggest that it is not essential.

LPS induces defence-related responses in plants

There have been numerous studies of the responses of plant to pathogens in terms of patterns of gene expression and synthesis of macromolecules and substances such as phytoalexins. A broadly consistent picture has emerged, although in most cases the defensive role of the molecules which accumulate is unclear. This is why the term 'defence-related' is used; to attribute a direct defensive function would be unjustifiable in many cases.

Several reports have described defence-related responses induced by LPS treatment of plants. *R. solanacearum* LPS induced a polypeptide of unknown function and also soluble peroxidase activity (Leach et al., 1983; Nadolny and Sequeira, 1980). The kinetics of accumulation of the former correlated with the development of LIR. LPS of *X. campestris* induced β-1,3-glucanase in *Brassica* (Newman et al., 1995), but LPS from *E. coli* did not, even at concentrations 100 times higher than that required for *X. campestris* LPS. Since both *X. campestris* and *E. coli* LPS can induce LIR, this result implies that the enzyme is not required for LIR. Other changes induced by LPS include induction of antimicrobial activity (Rathmell and Sequeira, 1975), induction of glyceollin synthesis in soybean (Barton-Willis et al., 1984) and changes in plant cell wall ultrastructure (Graham et al., 1977). Not all plant responses associated with defence are induced by LPS. Notably, the oxidative burst which is a characteristic feature of HR was not induced in soybean cultures by LPS. Moreover LPS did not block the oxidative burst induced in these cells by the elicitor polygalacturonate (unpublished work cited by Dow et al., 2000).

LPS pretreatment alters defence-related gene induction patterns

In addition to direct effects of LPS on gene expression, LPS pretreatment of pepper leaves altered patterns of gene expression induced by subsequent challenge with bacteria. Genes encoding the pathogenesis-related (PR) proteins P6, acidic β-1,3-glucanase and basic β-1,3-glucanase were not induced by *S. minnesota* LPS, but *X. campestris* LPS gave weak, transient expression (Newman et al., 2000). However pretreatment of pepper leaves with LPS from either source (under conditions which induced LIR) caused marked changes in the kinetics and degree of expression following subsequent challenge with *X.c. campestris* and *X.c. vesicatoria* (Newman et al., 2000). While the interpretation of such experiments must take into account the possible degradation of mRNA in tissue undergoing necrosis (i.e. tissue not pretreated with LPS), it is clear that LPS has specific effects on the induction pattern. The gene *HSR203J* isolated from tobacco is specifically induced at the onset of HR induction, although its function is unknown, In pepper, LPS pretreatment caused a more rapid induction of the *HSR203J* homologue following challenge (Newman et al., 2000). LPS can be said to have sensitized the leaf tissue so that it reacted more rapidly and strongly to bacterial challenge.

Production of phenolic compounds in LPS-treated leaves

Many studies have shown that salicylic acid (SA) is a signal molecule in the induction of a number of defence-related responses in plants, including HR, systemic acquired resistance and PR protein induction. However, induced systemic resistance triggered by treatment of roots of Arabidopsis with biocontrol bacteria (*Pseudomonas fluorescens*) does not involve SA (Pieterse et al., 1996). The possible role of SA in LIR has been examined in the *X. campestris*-pepper system (Newman et al., 2000). HR induced both by *X.c. campestris* and *X.c. vesicatoria(avrBs1)* was associated with increased levels of SA and its glycoside conjugate. LPS treatment alone did not induce SA accumulation. In LPS-pretreated tissue SA was

induced in response to bacterial challenge with a similar time course, but levels were fivefold less than in plants not pretreated. In compatible interactions with *X.c. vesicatoria* no SA accumulation was seen, with or without LPS pretreatment.

Another class of phenolic substances, the hydroxycinnamoyl tyramines, show characteristic patterns of accumulation and provide excellent molecular markers for LPS action (Newman et al., 2000). Incompatible interactions of pepper with *X.c. campestris* or *X.c. vesicatoria(avrBs1)* are characterized by accumulation of two hyroxycinnamoyl tyramines, coumaroyl tyramine (CT) and feruloyl tyramine (FT), together with unidentified flavonoids. LPS pretreatment caused these compounds to accumulate rapidly (2–4 h) after challenge inoculation whereas in untreated plants CT and FT did not begin to accumulate until 12 h. In compatible interactions much lower levels were induced, but again LPS pretreatment accelerated the accumulation. LPS treatment alone did not induce CT and FT accumulation. Previous suggestions that CT and FT have a role in plant defence (Grandmaison et al., 1993; Keller et al., 1996) are consistent with the induction patterns observed. CT and FT have growth inhibitory activity against *X.c. campestris*. As mentioned above, inhibition of bacterial growth (usually transient) can be observed in plants showing LIR. However, it is premature to conclude that CT and FT are alone responsible for growth inhibition and, indeed, there is evidence against this. LIR is only observed within a temporal window of 12–30 h separating LPS treatment and challenge inoculation. When the challenge inoculation was delayed so as to give a longer interval after LPS administration, LIR and concomitant growth inhibition were not seen, but the characteristic rapid accumulation of CT and FT still occurred.

In addition to occurring as free compounds, a substantial fraction of CT and FT becomes linked by esterification to plant cell polymers. The wall-bound fraction of CT and FT showed a rapid increase, similar to the free compounds, during LIR. It is possible that this modification of cell wall structure plays a role in resistance to pathogens, but this has not yet been studied.

CT and FT are synthesized by the condensation of coumaroyl or feruloyl CoA (derived from the phenylpropanoid pathway) with tyramine, a reaction catalyzed by tyramine-hydroxycinnamoyl transferase (THT) (Farmer et al., 1999; Negrel and Javelle, 1997; Schmidt et al., 1998; 1999). Tyramine is derived from tyrosine by the action of tyrosine decarboxylase (TyDC). Induction of CT and FT synthesis in pepper during an incompatible interaction with *X.c. campestris* was accompanied by an increase in transcription of genes encoding THT and phenylalanine-ammonia lyase (PAL), a key enzyme involved in production of phenylpropanoid compounds (Newman et al., 2000). Gene probes for TyDC in pepper are not available, but a transient increase in TyDC enzyme activity was detected after bacterial inoculation. LPS treatment of pepper gave an increase in THT gene transcription and TyDC enzyme activity, but PAL was not induced. Upon subsequent bacterial inoculation PAL was rapidly induced. Thus two of the three key enzymes of CT and FT synthesis are induced by LPS alone, but the third, PAL, requires inoculation with bacteria. It seems likely that this is the explanation of the apparent sensitization of the plant tissue by LPS, enabling rapid synthesis of CT and FT following bacterial challenge (Newman et al., 2000).

Understanding the biological role of LPS in plant–bacterial interactions

The evidence reviewed above indicates that bacterial LPS has specific effects on the interactions of bacterial pathogens with plants. In this final section, which is necessarily speculative, possible molecular mechanisms and the biological significance of the phenomenon will be considered. We noted above that, in experiments involving inoculation with live or heat-killed bacteria, it is not known in what form LPS is presented to plant cells. Intact LPS may be perceived as an integral part of the bacterial surface, or released in micelles or blebs (Beveridge, 1999). Alternatively, it is possible that portions of LPS may be released from bacteria following degradation by bacterial or plant enzymes, although we are unaware of any evidence which supports this. The first step in the chain of events is likely to be the interaction of LPS with a plant receptor. LPS receptors have been studied in relation to bacterial pathogens of animals (Darveau, 1998), but remain unexplored in plant biology. A receptor for a conserved domain of bacterial flagellin has recently been identified in Arabidopsis (Felix et al., 1999), and the binding of the ligand to the receptor initiates a chain of events. This may provide a model for a putative LPS receptor. The task of identifying a plant LPS receptor is likely to be difficult using biochemical approaches; genetic strategies may be more straightforward. By analogy with other defence responses, the perception of

LPS by the receptor is likely to generate a signal which is transduced by a series of proteins to effect the final biochemical responses. Genetic strategies have been very successful in identifying signal transduction components involved in gene-for-gene resistance systems to pathogens in plants (Feys and Parker, 2000). Using appropriate Arabidopsis mutants, experiments are in progress to determine whether known signal transduction components are involved in LIR, and similar experiments have been reported for induced systemic resistance (Pieterse et al., 1996; 1998). It is known that LPS treatment induced the expression of some defence-related genes. Modern microarray technology building upon genomic sequencing projects allows the whole set of genes of Arabidopsis to be quickly surveyed for changes in expression level following LPS treatment. Such experiments are in progress.

The most likely cause of LIR is the creation of an antimicrobial environment in the plant tissue. CT and FT may contribute to this. All the experiments described here have used bacterial pathogens to challenge LPS treated tissue. It would be interesting to know whether LIR is also effective against fungal and viral pathogens. However, there are technical difficulties to be overcome. The transient nature of LIR may make it difficult to observe inhibition with these pathogens which typically take longer than bacteria to produce symptoms. The reason for the transient nature of LIR is not known but may be caused by the turnover of essential components.

Future investigations of the mechanisms of LIR will use genetic strategies. LIR works well in Arabidopsis. The use of microarrays for studies of gene expression patterns following LPS treatment have already been mentioned. An advantage of Arabidopsis, which has been exploited in countless investigations, is the ease with which mutants defective in the process of interest can be isolated. In the case of LIR, mutants showing altered behaviour following LPS treatment and/or pathogen challenge would have the potential to reveal LPS receptors, signal transduction components and the final effectors of LIR. Such mutants could in principle be isolated by 'brute force' screening, but the project would be formidably labour-intensive. Thousands of individual plants from mutagenized stocks would have to be inoculated first with LPS and then with bacteria, and subsequently examined closely for LIR appearance. Macroscopic symptoms would also have to be related to bacterial survival in the tissue. A more promising approach could come from microarray expression studies. If genes could be identified which showed substantial changes in expression specifically in response to LPS treatment, it should be possible to devise a simpler screen for mutants based on appropriate reporter gene technology. As well as using induced mutants it would be of interest to explore natural genetic variation for LIR behaviour among the large number of wild Arabidopsis accessions which are available. If found, natural variation could provide clues about the biological significance of LIR. The results of a programme of genetic analysis of LIR should clarify the mechanisms involved and their relationship to other phenomena in plant–microbe interactions.

The conditions used to demonstrate LPS action and LIR are somewhat artificial, and it is necessary to consider the possible significance of LIR in natural conditions. In the laboratory, LPS solutions and bacterial suspensions are infiltrated into the intercellular spaces of leaves. It is not known which leaf cell types respond to LPS, and therefore whether LPS could indeed come into contact with responsive cells under natural conditions. While many bacterial pathogens normally gain entry to plants through stomata and thence colonize the intercellular spaces of leaves, others enter *via* hydathodes, nectaries, lenticels, etc, and colonize other tissues such as xylem. Whether LIR or equivalent phenomena occur in these situations is not known.

It is possible that LIR contributes to broad-spectrum resistance to saprophytic bacteria. Saprophytes may under certain conditions enter stomata and the intercellular fluids within leaves can support limited bacterial growth. The LPS of saprophytes is likely to be recognized by the plant cells and initiate LIR. If LIR can indeed contribute to resistance to saprophytes, it might be expected that virulent pathogens would have evolved mechanisms to minimize the effects of LIR. Several mechanisms could in principle achieve this. 1. The detailed structure of pathogen LPS may render it less active as a trigger of LIR. Although this has not been adequately studied, we have noticed that *X. campestris* and *E. coli* LPS have different specific biological activity in terms of induction of β-1,3-glucanase in turnip leaves (Newman et al., 1995). 2. Pathogens may have evolved to minimize presentation of LPS to plant cells, for example by masking of surface structures or by controlling LPS release in blebs. 3. Pathogens may be less susceptible to inhibition in LIR-expressing tissue. Little information is available to support or reject possibilities 2 and 3. 4. Pathogens may be able to suppress the development of LIR. The kinetics of accumulation of

CT and FT in tissue exhibiting LIR give some support to this concept. When *hrp* mutants of *X.c. campestris* were used to challenge LPS-pretreated pepper leaves the accumulation of the conjugates was much more rapid than during the corresponding challenge with wild type bacteria. This suggests that *hrp* gene action mediates partial suppression of the process. The local nature of LIR is consistent with a role in resisting colonization of the plant by saprophytic bacteria which gain occasional access to the internal spaces of leaves. The other characteristic feature of LIR is that it is temporary and can only be observed within a window of ca. 10–30 h in pepper. This implies that even a temporary setback to saprophytic invaders may be enough to protect the plant and also suggests that LIR imposes a metabolic 'load' on the plant, so that constitutive systemic expression of the inhibitory activity would be disadvantageous.

LPS-mediated LIR and similar phenomena are less well understood than gene-for-gene resistance mechanisms which lead to HR. Nevertheless LIR probably forms a significant part of the portfolio of defence mechanisms which plants use to combat infection. Further research is needed to unravel the complexities of the system and to lay the foundations for exploitation in crop protection.

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