Molecular analysis of the interaction between *Xanthomonas oryzae* pv. *oryzae* and rice

F.F. White, J.M. Chittoor, J.E. Leach, S.A. Young, and W. Zhu

Bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is the most devastating bacterial disease of rice in Asia. Future genetic strategies to improve resistance will benefit from a better understanding of the molecular events that mediate resistance in the interaction of host and pathogen. We have characterized two avirulence genes, *avrXa10* and *avrXa7*, which trigger resistance in rice lines carrying the resistance genes *Xa10* and *Xa7*, respectively. The two *avr* genes are members of an avirulence gene family that appears to be exclusive to *Xanthomonas*. In efforts to further understand the modes of action of the genes, the interdependence of *avr* and *hrp* gene function is being investigated. Activity of *avrXa10* requires a functional *hrp* regulon. The dependence does not appear to involve *avr* gene regulation or lack of bacterial growth in *hrp* mutants, and the *hrp* genes cannot rescue *avr* activity when supplied *in planta* by mixed bacterial strain inoculation. The results suggest that *avr* activity requires that both type of genes be present in the same bacterial cell. The *hrp* secretory pathway may be required for *avr* protein secretion or for secretion of an elicitor whose synthesis is catalyzed by *avr* protein products. At the same time, peroxidase and related host genes that are induced during a resistance reaction are being characterized. Cationic peroxidase PO-C1 was localized to xylem lumen and parenchyma cell walls at the site of infection by an incompatible strain of *Xoo*. Peroxidase transcript levels appear to peak within 12 h of challenge with bacteria.

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is the most devastating bacterial disease of rice in Asia (Ou 1985). Rice is a major target for crop improvement, and several strategies to improve disease resistance in rice through genetic engineering have been proposed (Toenniessen 1991). One approach involves the introduction of cloned disease resistance genes into rice cultivars (for review, see

Martin 1995). Blight has been controlled through the use of single-gene resistance introduced by traditional breeding methods. Genetically engineered resistance would expedite traditional breeding methods, facilitate the introduction of multiple resistance genes (pyramiding), and provide a wider range of sources for resistance genes. Although inexpensive and environment friendly, host-plant resistance has often been unstable. Instability of resistance to *Xoo* has been attributed to changes in the pathogen population as new virulent strains arise (Mew et al 1992). The use of genetic resistance will benefit from a better understanding of the molecular interactions between the pathogen and plant. Subsequent manipulation of the resistance genes may allow the design of more stable resistance.

Race specificity and *avr* genes

To identify critical factors involved in the elicitation of defense to bacterial invasion, we have been using molecular genetic analyses of both the pathogen and the plant. In interactions between *Xoo* and rice, resistance is governed by an interaction between single, dominant resistance genes (R genes) in rice and corresponding pathogen genes called avirulence (*avr*) genes (Mew 1987, Leach and White 1995). The products of *avr* genes control factors that elicit a plant resistance response. Races of *Xoo* are defined by the presence (or expression) of a unique combination of *avr* genes in the pathogen. Race 2, for example, should contain *avrXa10, avrxa5,* and *avrXa7*.

We have cloned and characterized two *avr* genes (*avrXa7* and *avrXa10*) from *Xoo* (Hopkins et al 1992). *Xoo* strains containing the cloned *avr* genes acquired the ability to elicit resistance when inoculated to rice cultivars with the corresponding resistance genes (*Xa7* and *Xa10*). The *avrXa7* and *avrXa10* genes are members of a gene family from *Xanthomonas* that are typified by the first cloned member of the family, *avrBs3* from *X. campestris* pv. *vesicatoria* (Bonas et al 1989). Strains of *Xoo* contain 12-14 copies of genes related to *avrXa7* and *avrXa10* (Hopkins et al 1992). To date, members of this gene family have been cloned from pathogens of such diverse hosts as pepper (Bonas et al 1989, Canteros et al 1991), cotton (De Feyter and Gabriel 1991, De Feyter et al 1993), citrus (Swarup et al 1991, 1992), and rice (Hopkins et al 1992). The gene family has not been identified in other genera.

The structure of the *avr* gene family from *Xanthomonas* is striking in that the middle third of the encoded protein of each member is a repeated sequence of 34 amino acids (Fig. 1). The number of repeats in an individual gene varies from 13.5 copies for *avrb6* from *X. campestris* pv. *malvacearum* to 25.5 copies in *avrXa7* (Bonas et al 1989, De Feyter et al 1993, Hopkins et al 1992), and are highly conserved with the exception of amino acids at positions 12 and 13. The sequence of positions 12 and 13 of each repeat is referred to as the variable region (Fig. 1). All members of the gene family encode relatively large polypeptides in the range of 120 kd. Although some intriguing structural features have been suggested based on the amino acid sequence (Yang and Gabriel 1995), no similarities to other proteins with known biochemical functions have been identified.

Repeat #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
avrXa10	NI	HG	NI	HG	NI	NI	NN	HD	NI	HD	NN	HG	NS	NG	HD	NG		
avrBs3	HD	NG	NS	NG	NI	NI	NI	HD	HD	NG	NS	NS	HD	HD	HD	NG	HD	NG
avrBs3-2	NI	NG	NI	NI	NG	NG	NI	NS	NG	NI	NS	NG	HD	HD	NS	HD	NG	NG
avrb6	HD	NI	NG	HD	HD	NI	HD	NI	NS	HD	HD	HD	NN	NG				

Fig. 1. ***avrBs3*** **gene family structure. Map of pBS*avr*Xa10 is shown as representative of the family. Amino acid repeat structure of the variable region for four of the members is shown below the map. Only the highly variable amino acids of the 12th and 13th codon of each of the 34 amino acid repeats are shown. Single letter code is used for amino acids. B, *BamH*I; E, *EcoR*I; K, *Kpn*I; P. *Pst*I, Sp, *Sph*I; X, *Xba*I.**

The arrangements of the variable regions of all characterized genes vary and appear to be critical features for the race specificity of the proteins. Some deletions in repeats of *avrBs3* resulted in the loss of avirulence activity on pepper plants containing the *Bs3* gene for resistance and, concomitantly, gained new avirulence activity on tomato (Herbers et al 1992). Upon replacement of the repeat region of *avrXa10* with the region of *avrXa7* and *avrBs3,* the resulting phenotype was found in our laboratory to confer specificity that corresponds to the source of the repeat domain (Fig. 2). The *avrBs3* repeat domain in *avrXa10* conferred a resistant phenotype to *X.c*. pv. *vesicatoria* on pepper containing the *Bs3* gene, and a hypersensitive reaction (HR) response to *Xoo*, which normally does not give an HR on the nonhost plant pepper, when inoculated on pepper (Fig. 2). The *avrXa7* repeat in *avrXa10* conferred specificity for the resistance gene *Xa7* and lost specificity for *Xa10* (Fig. 2).

Some homologs of *avrBs3* appear to have a role in aggressiveness or fitness of the pathogen. This property was first recognized for the *pthA* gene of *X. campestris* pv. *citri* and has been described for other members of the gene family (Swarup et al 1991, De Feyter et al 1993). The *avrXa7* gene of *Xoo* also appears to act in this manner. If *avrXa7* is inactivated, the mutant strains are less aggressive on a susceptible cultivar (Fig. 3). These results may help explain the retention of *avr* genes, in general, and, more specifically, the presence of many copies of the genes in *Xoo*. The introduction of new plant genotypes to control the pathogen may select for new versions of the gene family that avoid recognition for resistance and retain functions for aggressiveness.

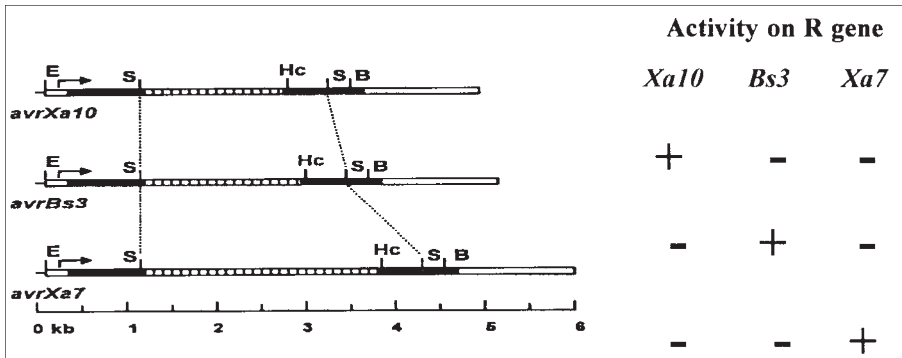

Fig. 2. Effect of repeat region on avirulence activity. The *Sph*I fragments of avirulence genes *avrBs3* and *avrXa7* were substituted for the *Sph*I fragment of *avrXa10*, introduced into *Xanthomonas* and tested on appropriate plant cultivars. The *avrBs3* construct was tested in both *X.c.* pv. *vesicatoria* and *Xoo. Xoo* without *avrBs3* is not pathogenic and does not elicit an HR on pepper. +, HR; -, pathogenic or no response.

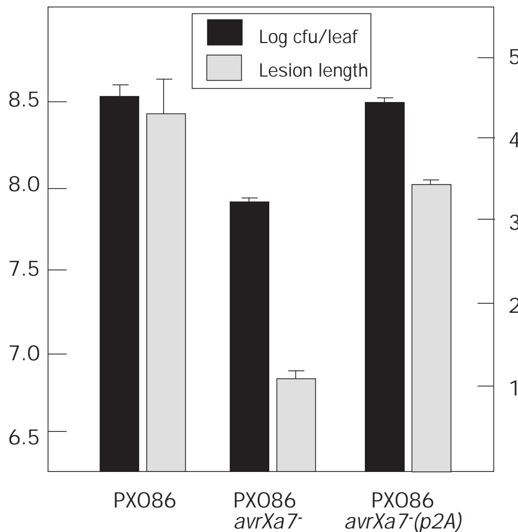

Fig. 3. Effect of loss of *avrXa7* activity in *Xoo*. Bacterial cell numbers (cfu; cell-forming units) and lesion lengths were measured after inoculation of rice cultivar IR24 with strain PXO86 and derivatives. Plasmid p2A contains a copy of *avrXa7* and was used for complementation of *avrXa7* mutation.

Avirulence gene function

Several models for avirulence gene function have been put forward to explain the molecular basis for induction of resistance in race-specific interactions (Leach and White 1995). The model that predicts the avirulence gene product as the elicitor of resistance would appear to be the simplest explanation for the *avrBs3* family (Fig. 4, Model A). If this model operates in the *Xoo*/rice interactions, the products of the *avr* genes would be secreted outside the bacterial cells where they could interact with the plant cell. A consensus signal peptide has not been found at the amino terminus of any of the *avrBs3* family members. Nor has the product of the *avrXa10* gene, like that of *avrBs3* (Brown et al 1993), been shown to be located extracellularly (Young et al 1994). In addition, neither the *avrXa10* gene product, which was purified from *E. coli* or *Xoo* cells, nor the extracellular fluids of rice leaves undergoing an incompatible response elicited resistance in rice plants containing *Xa10* (Young et al 1994).

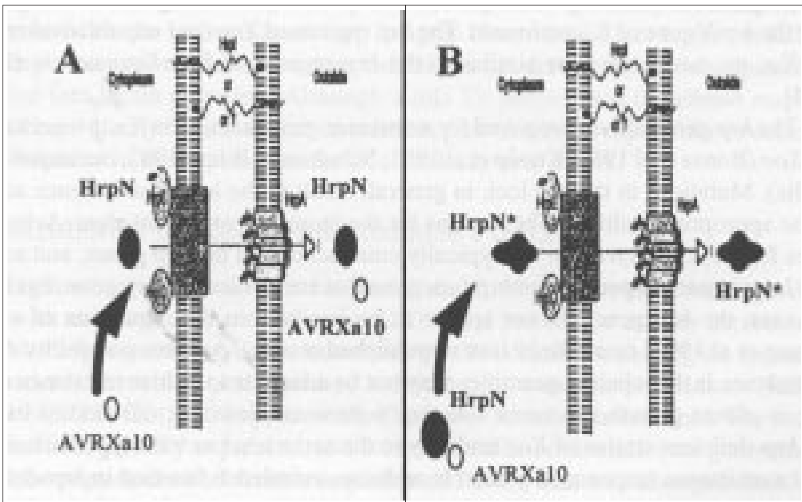

Fig. 4. Models for *avrXa10* and related gene function in avirulence. A, the *avrXa10* gene product or some processed form is secreted extracellularly via *hrp* secretory apparatus. B, the *avrXa10* gene product modifies a harpin-like protein, which is in turn secreted by *hrp* secretory apparatus. Schematic of *hrp* apparatus is modified from Van Gijsegem et al (1994).

The *hrp* gene cluster and avirulence activity

The evidence that *avrXa10*-like gene products do not function outside the bacterial cell, although compelling, remains inconclusive. Bacterial proteins can be exported through alternate secretion pathways without the need for signal sequences (Salmond and Reeves 1994), and some exported proteins require modification for activity (Hughes et al 1992). Thus, the *avr* gene products may function as elicitors and may not have been detected outside the bacterial cells. In future studies, purification of the race-specific elicitors may be facilitated by artificially induced growth conditions that mimic the *in planta* conditions.

One class of genes likely to be involved in the production of the race-specific elicitors from *Xoo* are the *hrp* (*h*ypersensitive reaction and *p*athogenicity) genes. The *hrp* genes are known to be involved in the production of non-race-specific protein elicitors (elicitors not associated with race-specific or single gene resistance) (Bonas 1994). Three extracellular protein elicitors whose synthesis and secretion are directed by *hrp* gene clusters have been identified from *Erwinia amylovora* (Wei et al 1992), *Pseudomonas syringae* pv. *syringae* (He et al 1993), and *Pseudomonas solanacearum* (Arlat et al 1994). The first two proteins have been given the generic name harpin after the *hrpN* gene of *E. amylovora.* The *hrp* regions of *Xoo* (our unpublished results) and *X.c.* pv. *vesicatoria* are similar to the *hrp* regions of *P. solanacearum* (Bonas 1994).

The *hrp* genes also are required for avirulence gene function in *X.c.* pv. *vesicatoria* and *Xoo* (Bonas et al 1989, Knoop et al 1991, Schulte and Bonas 1992, our unpublished results). Mutations in the *hrp* loci, in general, result in the loss of avirulence activity on the appropriate cultivar. The reasons for the requirement are not clear. Avirulence genes from *P.s.* pv. *syringae* are typically coinduced with the *hrp* genes, and activity is, at least in part, dependent on *hrp* functions that are involved in *hrp* gene regulation. However, the *hrp* genes do not appear to be involved in the regulation of *avrBs3* (Knoop et al 1991) or *avrXa10* (our unpublished results). Another possibility for the dependence is that elicitor quantities may not be adequate to induce resistance due to poor *in planta* growth of strains with *hrp* mutations. However, our studies indicate that *hrp*-deficient strains of *Xoo* multiply to the same level as wild-type bacteria that elicit a resistance response in plants. In addition, avirulence function in *hrp*-deficient bacteria is not restored by growing the mutants in the presence of *hrp*+ bacteria. Thus, inadequate bacterial multiplication does not account for the lack of elicitor activity, and *avrXa10* and related genes need to be in cells with a functional *hrp* cluster.

In general, the *hrp* genes are induced *in planta* (Bonas 1994), and we are attempting to characterize the transcriptional control mechanisms to induce *hrp* function *ex planta.* In *P. solanacearum, hrp* gene transcription is directly or indirectly under the control of the *hrpB* gene, which codes for a homolog of the AraC/XylS family of transcriptional regulators (Genin et al 1992). Mutations in *hrpB* eliminate expression of *hrp* genes in *P. solanacearum.* Independently, a homolog of the *hrpB* was found in *Xoo* and was termed *hrpXo* (Kamdar et al 1993). The involvement of *hrpXo* in the transcriptional regulation of *hrp* genes in *Xoo* was not determined. We

have constructed a β-glucuronidase fusion with the reporter of the *hrpC* operon of *Xoo* and are investigating the role of *hrpXo* in the regulation of *hrp* transcription. We hope that manipulation of culture components and/or constitutive expression of the transcriptional regulator will allow expression of the *hrp* genes in liquid culture and facilitate the control of conditions for elicitor purification.

Defense response of rice to *Xoo*

Xoo is primarily a vascular pathogen that enters the plant through hydathode water pores or wounds (Tabei 1977, Mew et al 1984). Antibacterial compounds have been isolated from healthy leaves of susceptible and resistant rice cultivars, some of which were oxidized lignin components with aldehyde and phenol groups (Horino and Kaku 1989). Lignin polymers accumulate in inoculated leaves during the resistant interaction between rice cultivars carrying the *xa5, Xa7*, and *Xa10* genes for bacterial blight resistance and strains of *Xoo* carrying the corresponding avirulence genes (Reimers and Leach 1991). Lignin and other phenolic polymers serve as physical barriers (Ride 1983) and, as such, probably would not be an effective defense against *Xoo*, a vascular pathogen, unless lignified materials prevented bacterial spread by blocking vessels. Peroxidases are the last enzymatic step in lignin biosynthesis, that is, the oxidation of hydroxy cinnamyl alcohols into free radical intermediates, which subsequently are coupled into lignin polymers. Although a role for peroxidases in defense responses has not been clearly demonstrated, increases in peroxidase activity have been correlated with infection in many plant species (Kolattukudy et al 1992).

Localization of the cationic peroxidase PO-C1

After infiltration of rice cultivars containing the *Xa10* gene for bacterial blight resistance with strains of *Xoo* containing *avrXa10,* total peroxidase activity increased and several changes in the peroxidase isoenzyme profile occurred, including the appearance of a cationic peroxidase and increased activities of two anionic peroxidases (Reimers et al 1992). PO-C1 (pI 8.6, apparent M_r 43 kd) was further characterized. The enzyme was purified, partially sequenced, and domain-specific antibodies were generated to a synthetic peptide derived from a sequence of PO-C1 that was diverged from other plant peroxidases and shown to be specific for PO-C1 (Young et al 1995). Immunolocalization studies provided evidence that PO-C1 is located in the xylem vessels. First, PO-C1 accumulates in guttation fluids of resistant plants within 24 h of exposure to avirulent *Xoo* strains and was not detected in guttation fluids of plants undergoing susceptible interactions until a later time (48 h). Second, anti-PO-C1 antibodies were observed on the cell walls and lumen of xylem vessels within 24 h after inoculation with avirulent strains of *Xoo* (Fig. 5). Induced PO-C1 also was detected in the parenchyma cells adjacent to the xylem vessels, suggesting that the isoenzyme is expressed in neighboring cells and distributed to the anucleated xylem vessels. The accumulation and location of PO-C1 in xylem elements are consistent with a role for the peroxidase in rice defense responses against *Xoo*.

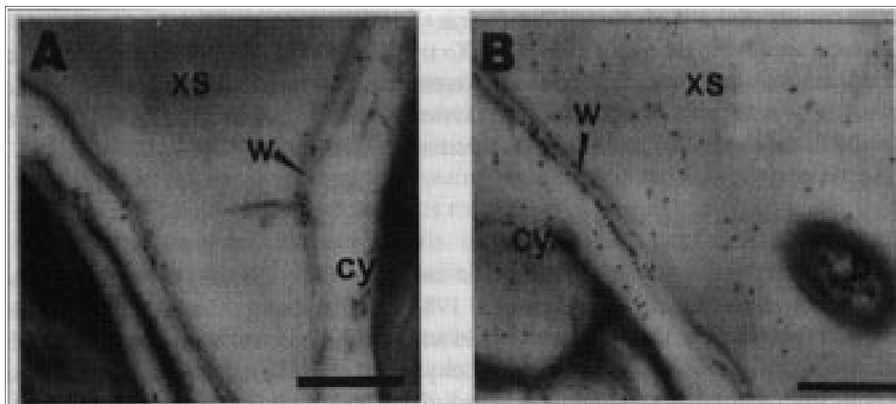

Fig. 5. Localization of PO-C1. Immunogold labeling of cationic peroxidase PO-C1 at 24 h after infiltration during (A) compatible and (B) incompatible reaction of rice and *Xoo*. w, cell wall; xs, extracellular space; cy, cytoplasm. Bar = 0.5 mm.

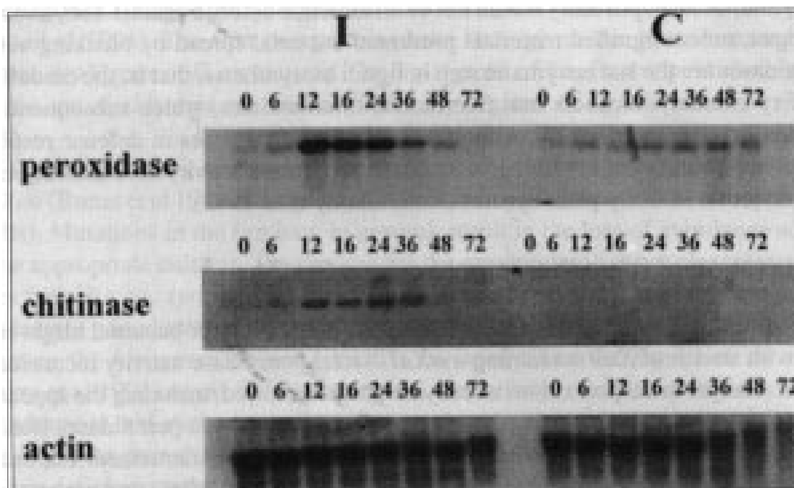

Fig. 6. Defense gene induction. mRNA was isolated from incompatible (I; resistant) and compatible (C; susceptible) interactions of *Xoo* and rice at the hours after inoculation as indicated above the lanes. The mRNA was subjected to electrophoresis, blotted, and hybridized with probes for peroxidase (3' end of OSPER, Reimmann et al 1992); chitinase (Anuratha et al 1992); and actin (McElroy et al 1990).

As part of the characterization of the peroxidase isozymes that are induced during the rice/*Xoo* interaction, cDNAs from resistant and susceptible interactions are being isolated. In conjunction with increases in peroxidase activity, mRNA corresponding to peroxidase increases to peak levels at 6 h in the incompatible (resistant) reaction. Peroxidase mRNA also increases in compatible reactions. However, peak activity levels are lower and occur at 36 h (Fig. 6). Messenger RNA of peroxidase and

other genes begins to degrade in the compatible reactions by 48 h. Chitinase (a defense-associated protein) mRNA levels increase up to 24 to 36 h, yet do not appear in the compatible response (Fig. 6). A variety of cDNAs that correspond to the mRNA in the different interactions have been or are in the process of being isolated. Hybridization analysis using the 3' end of each cDNA will allow further delineation of the expression pattern for each gene.

Summary and future directions

The avirulence genes that have been identified from *Xoo* are multicopy members of a gene family, and many questions remained regarding their role in the *Xoo*/rice interaction. Some homologs of the *avr* genes may correspond to R genes in rice other than *Xa10, Xa7*, or *xa5*. The *Xa21* gene recently cloned from rice contains features in common with other R genes (P. Ronald, University of California, Davis, 1995, pers. commun.), and is a particularly interesting case in point. The broad-spectrum resistance of the gene may be due to recognition of one or several of the *avrXa10* homologs. Identification of the avirulence gene or genes that correspond to *Xa21* will provide a better understanding regarding the stability and utility of the gene.

The mode of action of most avirulence genes including the *avrBs3* family remains unknown. Despite the results of the localization studies, it remains reasonable to speculate that *avrXa10* and related gene products are the elicitor molecules that interact with the resistance gene product in a signal transduction complex. Our current direction is to determine the interaction of the *avr* gene products with *hrp* genes, and whether some portion of the *avr* protein is externalized via the *hrp* secretory apparatus. An additional possibility is that the protein is directly transferred to the plant cell and never occurs in the external growth medium (Yang and Gabriel 1995). If supportive evidence for the hypothesis is found, physical alterations in the avirulence protein will be correlated with changes in elicitor activity, binding and, in the case of *avrXa7,* aggressiveness. An understanding of the interaction between elicitor and R proteins may allow the engineering of new and more stable resistance.

An alternative approach to introducing R genes for rice improvement involves the introduction or enhanced expression of genes whose products either inhibit pathogens directly (e.g., lysozyme, antimicrobial peptides, proteinase inhibitors) or are involved in the biosynthesis of toxic compounds (Toenniessen 1991). The success of these approaches will depend on expression and targeting the antimicrobial enzymes to the proper tissue. Perhaps even more important, new and more effective strategies will be evident as we clarify the physiological and molecular events of resistance. The characterization of peroxidase will provide both insight regarding the functions required for resistance, as well as information on how enzymes are targeted to the diseased tissue.

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Notes

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