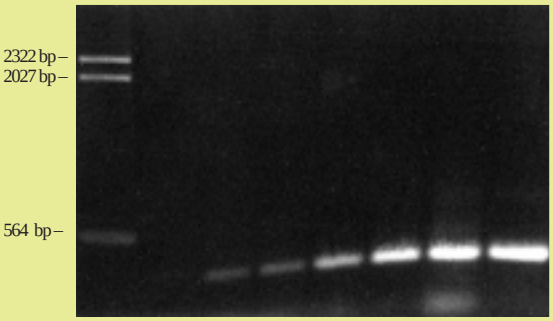

Figure 4 Agarose gel electrophoresis of a second PCR amplification of products obtained with primers 1 and 2 from various numbers of zoospores of isolate 149 of *Phytophthora nicotianae* fixed on a nitro-celulose membrane: Lane 1 - DNA marker size *λ/HindIII*; lanes 2 to 7 respectively - zoospore numbers 0,10^2,10^3,10^4,10^5,10^6; lane 8 - amplification product of a single PCR on pure fungal DNA (isolaote149).

Two successive cycles were required to obtain a signal, even when one million zoospores were fixed to the membrane. However, with a second round of PCR, a signal was observed with just one hundred zoospores, and the intensity of the signal increased with increasing number of zoospores (Fig. 4). When one million zoospores were present, the signal was identical to that obtained when a single PCR was made with pure fungal DNA. A high annealing temperature during the second round of PCR increased the specificity of DNA amplification. As expected, a negative control of water lacking zoospores gave no signal after the two cycles of PCR.

Whereas ELISA-based techniques detected as few as 10 zoospores, a *P. nicotianae*-specific amplification signal was obtained with an estimated 100 zoospores trapped on a membrane, although the efficiency of the trapping has not yet been determined. Our protocol therefore may yet require further refinement. However, these results offer a realistic alternative to the use of ELISA-based tests for the detection of *P. nicotianae* in plants and as zoospores. Given that all species of *Phytophthora* probably have equivalent elicitin gene sequences, PCR-based diagnostic techniques (detection and identification) based on elicitin genes could be developed for all species in the genus.

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Isolation of genes encoding polygalacturonase-inhibiting proteins from raspberry

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Ripe raspberry fruits are highly susceptible to grey mould disease, caused by the fungus *Botrytis cinerea*, and the losses due to the disease after harvest are a major factor limiting the sale of fresh fruits on distant markets and the quality of processed fruits. *B. cinerea* attacks the flowers of raspberry as soon as they open and establishes a quiescent infection in the styles and other floral organs, but the disease does not appear until fruits ripen. Biochemical studies *in vitro* have shown that *B. cinerea* produces endo-polygalacturonases (PGs) which degrade polygalacturonic acid, the 'backbone' of the large pectin molecule in the primary walls and intercellular matrix of fruit tissues (Ann. Rept. 1992, 70-72).

A raspberry polygalacturonase-inhibiting protein (PGIP) purified from immature green fruits inhibits *Botrytis* endo-PG activity; this activity declines sharply with the onset of ripening which corresponds with an increase in susceptibility to infection. PGIPs

Cons	P	-	-	-	-	-	L	-	-	L	-	-	L	-	L	S	-	N	-	L	-	G	-	I
LRR1	E	C	D	P	T	T	H	R	I	N	S	L	T	I	F	T	D	N	N	L	T	G	Q	I
LRR2	P	A	Q	V	G	A	L	P	Y	L	E	T	L	E	L	RK	L	P	H	L	T	G	P	I
LRR3	Q	P	S	I	A	K	L	K	H	L	K	M	L	R	L	S	W	N	G	L	S	G	S	V
LRR4	P	D	F	I	S	Q	L	K	N	L	T	F	L	E	L	N	F	N	K	F	T	G	S	I
LRR5	P	S	S	L	S	Q	L	P	N	L	G	A	L	H	L	D	R	N	Q	L	T	G	Q	I
LRR6	P	S	S	F	G	K	F	V	G	T	VP	A	L	F	L	S	H	N	Q	L	T	G	K	I
LRR7	P	T	S	F		A	N	M	N	F	D	Q	I	D	L	S	R	N	K	L	E	G	D	A
LRR8		S	V	I	F	G	L	N	K	T	T	Q	IV	D	L	S	R	N	M	L	E	F	D	L
LRR9		S	K	V	V	F	S	T	S	L	R	A	V	D	L	N	H	N	S	I	T	G	S	I
LRR10	P	A	Q	L	T	Q	L	D	D	L	V		LF	N	V	S	Y	N	R	L	C	G	K	I

Figure 1 Alignment of leucine rich repeats in raspberry PGIP1. Tandem leucine Rich Repeats (LRR1-10) extend from amino acid 63 to amino acid 302 and are aligned in comparison to the consensus (cons) derived for previously characterised PGIPs.[1]

have been purified from a number of plant families and recently the genes encoding some of them have been published (see Ann. Rept. 1994, 65). One strategy to improve resistance to *Botrytis* infection is to increase the levels of active PGIP in ripening fruit by activating expression of PGIP genes.

Structure of PGIP genes Two PGIP genes have been isolated from a raspberry fruit cDNA library. PGIP1 contains an open reading frame encoding a 331 amino acid protein. The deduced amino acid sequence shows a high degree of similarity with previously isolated PGIPs (Table 1), and contains features characteristic of PGIPs found in several plant families. PGIP1 contains an N-terminus of 22 amino acids which targets the peptide for export through the cell membrane to its final location in the plant cell wall. Four cysteines towards the N-terminus of the mature peptide and four cysteines towards the C-terminus are probably required for correct folding of the extracellular peptide to its final structure as a molecule capable of interacting with fungal endo-PGs. PGIPs are glycosylated and the raspberry PGIP contains four N-glycosylation sites (N-T/S), three of which show a conserved position with previously isolated PGIP genes. Variation in the glycosylation of PGIPs may modulate the wide differences in activity and specificity reported in purified PGIPs from different plants.

	Amino acid % similarity	
	PGIP1	PGIP2
Pear	86.8	76.1
Kiwifruit	77.3	71.5
Tomato	77.2	67.3
Antirrhinum	71.8	62.8
Bean	66.0	56.5
Soybean	61.3	58.2
Raspberry PGIP2*	88.5	-

*Raspberry PGIP2 is compared to the other sequences up to the frame-shift mutation

Table 1 Comparison of raspberry PGIP peptide with published peptide sequence.

Leucine-rich repeat motifs PGIP1 shows a high leucine content (15.7%) and contains 10 loosely conserved leucine-rich repeat (LRRs) motifs (P-----L--L--L-LS-N-L-G-I) (Fig. 1). LRRs have been described in several recently isolated plant resistance genes. The LRRs of PGIPs have a high homology to the *Cf9* and *Cf2* genes of tomato, conferring major gene resistance to the fungal pathogen *Cladosporium fulvum*. LRRs are a key feature in other protein-to-protein interactions, such as the characteristic non-globular structure of the porcine ribonuclease inhibitor and its molecular interaction with RNase A.

PGIP2 may be a pseudogene. Comparison of PGIP1 and PGIP2 shows 88% similarity at the amino acid level (Table 1) and preserves all of the characteristics of PGIPs at the amino acid level. However, a single nucleotide insertion puts the coding sequence out of frame, leading to premature termination 226 amino acids downstream from the translation start.

Southern blotting shows PGIPs in raspberry to be part of a low copy number gene family. Expression analysis by reverse transcriptase-PCR and Northern blotting shows consistent levels of expression in flowers, immature and mature fruit. Since earlier biochemical studies showed that the activity of the enzyme inhibitor extractable from cell walls declined rapidly as fruits ripened (Ann. Rept. 1992, 70-72) the expression analysis suggests that post-translational modification may be occurring.

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