Inhibition of bacterial and fungal plant pathogens by thionins of types I and II

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Abstract

Thionins are cysteine-rich, 5 kDa polypeptides which are active against plant pathogens. Thionins of type I, from the endosperms of wheat (Wα1, Wα2, Wβ) and barley (Bα, Bβ), and of type II, from barley leaves (BLa, BLb, BLc), have been purified to apparent homogeneity. For a given pathogen, the effective concentration giving 50% inhibition (EC-50) determined for the different thionins varied over a less than fivefold range. The ranking of the variants according to their activity differed among different pathogens, but certain variants, such as Wα1, Wβ or Bβ, tended to be more active than the others. Strains of some bacterial species, such as *Clavibacter michiganensis* subsp. *sepedonicus* or *Pseudomonas solanacearum* were sensitive in the $2-3 \times 10^{-7}$ M concentration range, whereas the most sensitive fungal pathogens, such as *Rosellinia necatrix*, *Colletotrichum lagenarium* and *Fusarium solani*, had EC-50 values in the $1-4 \times 10^{-6}$ M range. Thionins, which were not particularly effective in liquid medium against *Phytophthora infestans* (EC-50 = 3.9×10^{-5} M) were more effective than the fungicide Ridomil on a molar basis in a drop application assay on leaf discs from potato.

Key words: Thionins; Plant pathogens; *Triticum aestivum*; *Hordeum vulgare*

1. Introduction

The thionins are among the first plant protein families for which a defense role has been proposed, based on their in vitro activity against plant pathogenic bacteria [1]. These cysteine-rich, 5-kDa polypeptides have been characterized in different tissues from wide-ranging taxa, and five structural types (I–V), with several variants from each, have been found (for reviews, see refs. [2–4]). Three of these types have been identified in wheat and barley: type I, which corresponds to the original endosperm thionins [5,6]; type II, characterized in etiolated barley leaves [7,8]; and type V thionins, whose primary structure has been recently deduced from cloned endosperm cDNA [9].

Crude preparations of wheat endosperm thionins (type I) had been found to be active against plant pathogenic bacteria of the genera *Pseudomonas, Xanthomonas, Agrobacterium, Erwinia* and *Clavibacter* at concentrations ranging from 1×10^{-7} M to 1×10^{-5} M, and a comparison of purified β-thionin with a mixture of α1- and α2-thionin seemed to show differential activity towards *Xanthomonas campestris* pv. *phaseoli* (Smith) Dye and *Pseudomonas solanacearum* (Smith) Smith [1]. Bohlman et al. [10] have reported that two phytopathogenic fungi, *Chalara paradoxa* (De seyn) Sacc. and *Drechslera teres* (Sacc.) Shoem., were sensitive to crude preparations of thionins of types I and II at a concentration of 5×10^{-4} M. Apart from these studies, which were carried out with crude thionin preparations or with thionin mixtures, the in vitro antipathogenic properties of pure thionin variants have not been reported and possible activity changes associated with the different variants have not been investigated. This information should be of help in the selection of appropriate plant/pathogen combinations for testing the possible protection effect of transgenically expressed thionin genes. The feasibility of such studies has been shown in our recent report of enhanced resistance to bacterial pathogens in transgenic tobacco expressing α-thionin (type I) from barley [11]. We now report a simplified method which has allowed the purification for the first time of type-II thionin variants, as well as that of previously known type-I variants, and a survey of the susceptibility of different bacterial and fungal pathogens to thionins.

2. Materials and methods

2.1. Microbial strains

Bacterial pathogens *Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann and Kottoff) Davis et al. Strain C5 and *Pseudomonas solanacearum* (Smith) Smith strain P2 were from the ETSIA collection (Madrid); *Xanthomonas campestris* pv. *oryzae* (Ishiyama) Dye and *Pseudomonas syringae* pv. *lachrymans* (Smith and Bryan) Young et al. were from CIBA GEIGY (Basel). Fungal pathogens *Rosellinia necatrix* Prill., *Fusarium solani* (Martius) Sacc., *Fusarium* sp. strains 72 and 78, *Botrytis cinerea* Pers. strains B100 and BC1 and *Rhizoctonia solani* Kühn were from the ETSIA collection (Madrid); *Colletotrichum lagenarium* (Pass.) Ell and Halsted, *Monilinia fruticola* (Winter) Honey, *Pythium ultimum* Trow, *Phytophthora infestans* (Mont.) de Bary, *B. cinerea*, *Septoria nodorum* (Berk.) Berk., *R solani*, *Pyricularia oryzae* Briosi and Lavara and *Erysiphe graminis* Dc. were from CIBA GEIGY (Basel). Nonpathogenic fungi tested, *Trichoderma viride* Pers. ex Gray strain CSA37 and *Aspergillus nidulans* (Eidam) Wint were from the ETSIA collection (Madrid).

2.2. Growth conditions for bacterial and fungal inocula

Inocula for the inhibition experiments were grown in the media and under the conditions summarized in Table 1. Bacteria were grown on slanted nutrient agar and removed by shaking the culture with 1 ml of nutrient broth (Oxoid). Bacterial density was measured spectrophotometrically at 600 nm and the suspension adjusted to the appropriate concentration with nutrient broth (Oxoid). All fungi were grown in 9 cm Petri dishes. To remove the spores, 3 ml of sterile water were added and the surface of the mycelium was gently scraped with a loop. When mycelium pieces were used as inoculum (*R. necatrix* and *R. solani*), homogeneization was carried out on a Potter homogeneizer. Spores or mycellium pieces were filtered through glass fiber prior to counting in a Thoma cell. Suspension were then diluted to the appropriate concentrations with the medium indicated in Table 2.

2.3. Purification of thionins

Crude thionin preparations were obtained from the endosperms of hexaploid wheat, *Triticum aestivum* L. cv. Candeal, and diploid barley, *Hordeum vulgare* cv. Betzes, by petroleum-ether extraction and HCl/ethanol precipitation, as previously described [12]. Mixtures of genetic variants of type I thionins were obtained from these preparations by preparative electrophoresis on 10% polyacrylamide columns (1.5×10 cm) with 0.1 M acetic acid buffer, pH 2.9, at 20 V/cm, essentially as reported [13]. Resolution of type I genetic variants

Table 1
Growth conditions for bacterial and fungal inocula

Species	Medium[a]	Duration	Conditions
Bacteria			
C.m. subsp. *sepedonicus* strain C5	NA	1 day	28°C, in the dark
P. solanacearum strain P2	NA	1 day	28°C, in the dark
X. campestris pv. *oryzae*	NA	1 day	25°C, in the dark
P. syringae pv. *lachrymans*	NA	1 day	25°C, in the dark
Fungi			
R. necatrix	PDA	7 days	22°C, in the dark
T. viride strain CSA37	PDA	7 days	22°C, in the dark
A. nidulans	PDA	8–9 days	22°C, in the dark
C. lagenarium	PCA	14 days	22°C, in the dark
Fusarium sp.	PDA	7 days	22°C, in the dark
M. fruticola	AP	4–6 days	22°C, in the dark
B. cinerea	PA	14 days	22°C, in the dark
P. ultimum	CB	21 days	18°C, in the dark
P. infestans	V-8	14 days	18°C, in the dark
		+7 days	15°C, in the dark
F. solani	5% SMA	7 days	22°C, in the dark
S. nodorum	CDV-8	8–9 days	18°C, constant black light[b]
P. oryzae	RPA	8–9 days	22°C, constant white light[c]

[a]NA, nutrient agar (Difco); PDA, potato dextrose agar (Difco); PCA, potato carrot agar [16]; AP, apricot medium [17]; PA, pea agar [16]; CB, carrot broth [16]; V-8, v-8 medium [18]; SMA, Sabouraud maltose agar (Difco); CDV-8, cdv-8 medium [19]; RPA, rice polish agar [20].
[b]Philips TL 40W/08F 40T12 Lamp.
[c]Philips TLMF 40W 33RS Lamp.

was achieved by reverse-phase-high-performance liquid chromatography (RP-HPLC), on an Ultrapore C3 column (1 cm × 25 cm; particle 5 μ; pore 300 A) from Beckman, using H_2O/2-propanol gradients, 0.1% trifluoroacetic acid, (0.5 ml/min), as indicated in Fig. 1.

Type-II thionins were extracted from etiolated barley leaves (7 days) with 0.1 M Tris–HCl buffer, pH 7.5, 10 mM EDTA, and precipitated from the extract by $(NH_4)_2SO_4$, between 35% and 55%, w/v, essentially as described [14]. The precipitate was redissolved in 0.1 M acetic acid and subjected to gel filtration on Sephadex G-25, using the same solvent, and the exclusion peak was freeze-dried. This material was fractionated by RP-HPLC as described above, using the gradient indicated in Fig. 1.

Apparent molecular weights of the purified proteins were determined by sodium-dodecyl-sulphate-polyacrylamide-gel-electrophoresis (SDS-PAGE) in preformed gradient gels (BioRad) according to the manufacturers instructions. Starch gel electrophoresis (SGE), in aluminum lactate buffer, at pH 3.2, and in Tris–HCl buffer, at pH 8.3, were carried out in the presence of 3 M urea as previously described [12].

Amino acid analysis was carried out according to the manufacturers instructions on an HPLC Beckman System and N-terminal amino acid sequencing was carried out by automated Edman degradation (Applied Biosystems).

2.4. In vitro inhibition tests

Thionins were dissolved in water as required. Bacteria were inoculated at a final concentration of 1×10^6 cfu/ml or 1×10^4 cfu/ml, in sterile microtiter plates at final volumes of 150 μl (100 μl thionin + 50 μl nutrient broth, Oxoid). Test conditions for the different fungal strains are indicated in Table 2. After 1–2 days of incubation at 28°C

Table 2
In vitro activity of purified thionin

Species	Conditions[a]	Thionins		EC-50 (μM)
		Tested	Most active	
Bacteria				
C. m. subsp. *sepedonicus* strain C5		I,II[b]	Wα_1	0.2
P. solanacearum strain P2		Wα_2,Wα_2,Wβ,II	Wβ	0.3
X. campestris pv. *oryzae*		I	Bβ	1.5
P. syringae pv. *lachrymans*		I		>4.0
Fungi				
R. necatrix	A	I	Wβ	1.5
T. viride strain CSA37	A	I,II	Wβ	2.0
A. nidulans	A	Wα_1 + Wβ		3.0
C. lagenarium	B	I		<4.0
F. solani	A	I,II	Wβ	4.0
Fusarium sp. strain 72	A	Wα_1 + Wβ		18.0
Fusarium sp. strain 78	A	Wα_1 + Wβ		10.0
M. fruticola	C	I	Wβ	<12.0
B. cinerea strain BC1	A	Wα_1 + Wβ		18.0
B. cinerea strain B100	A	Wα_1 + Wβ		25.0
B. cinerea (Basel)	C	I	Wα_2,Wβ,Bβ	40.0[c]
P. ultimum	B	I	Wα_1	26.0
P. infestans	C	I	Wβ	39.0
R. solani (Madrid)	A	Wα_1 + Wβ		35.0
R. solani (Basel)	C	I		>500.0
S. nodorum	C	I		250.0
P. oryzae	C	I		>500.0

[a]The following conditions were used: A, final volume of 60 μl (20 μl thionin solution + 40 μl liquid Czapek Dox). B, Final volume 100 μl (10 μl thionin + 90 μl liquid Czapek Dox). C, Final volume 100 μl (10 μl thionin + 90 μl pea seed medium). In the first case 10^6 spores or mycelium pieces/ml were used whereas in cases B and C, 10^4 spores/ml were used.
[b]I, all purified type I thionins; II, all purified type II thionins.
[c]Less than 50% inhibition at this concentration.

for bacteria and 2–3 days at 18°C–22°C for fungi, growth was recorded by measuring absorbance at 492–595 nm in an ELISA plate reader.

2.5. In vivo inhibition tests

For the leaf-disk assay with *P. infestans*, disks (1.5 cm in diameter) were cut out of potato leaves (var. Bintje) and floated with the underside upside down on 5 ml water in small Petri dishes, 6 disks per dish. Thionin (Wα_1, Wα_2, Wβ, Bα or Bβ), Ridomil or water (control) were added on the middle of the disks as a 10-μl drop. Two hours later, disks were inoculated with a 10-μl drop of a suspension containing about 500 zoospores of *P. infestans*. The 'infection drop' was deposited at the same place as the 'treatment drop'. The dishes were incubated at 18°C and the surface of the disks covered with mycelium was evaluated 5 days later by visually estimating the percentage of surface covered with mycelium (mean of six replicates).

For the tests on apples with *B. cinerea*, 3 small holes (5 mm in diameter and 3 mm deep) were dug in the apple and 50 μl of the thionin solution (Wα_1, Wα_2, Wβ, Bα or Bβ) was deposited in the holes. One hour later, 50 μl of a spore suspension of *B. cinerea* (5×10^5 spores/ml) were deposited at the same place and the apples were incubated for 5 days at 20°C in the dark. The diameter of the fungal colonies on the apples was then measured.

In the tests on barley with *E. graminis*, the

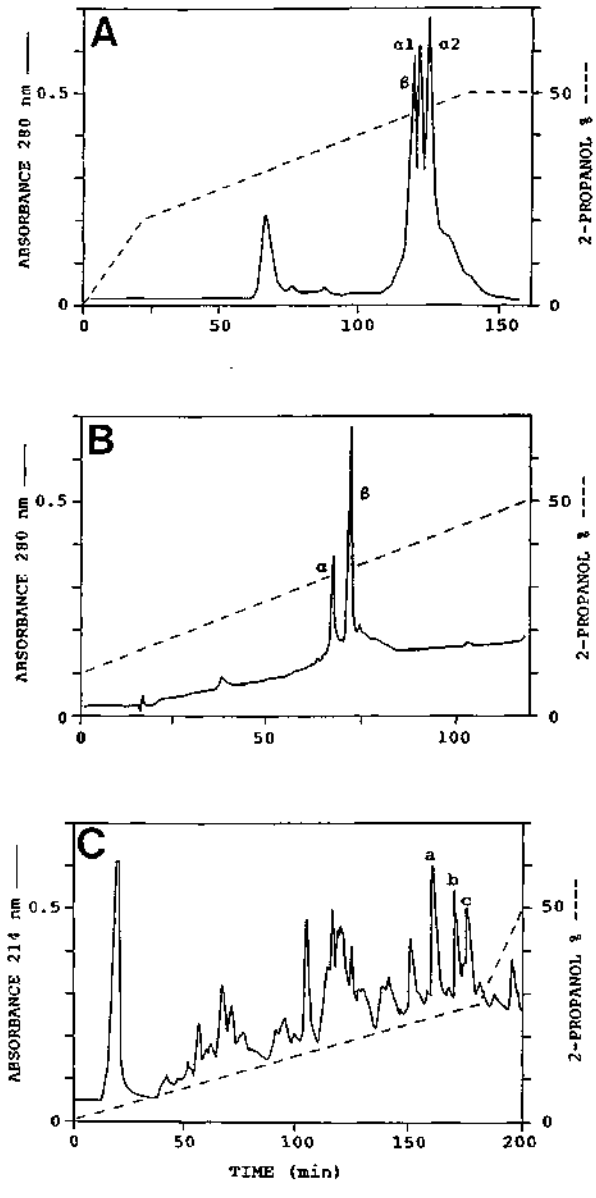

Fig. 1. Purification of thionins of types I and II. A, RP-HPLC fractionation of a thionin preparation obtained by preparative electrophoresis from a crude extract from wheat endosperm. Variants $W\alpha_1$, $W\alpha_2$ and $W\beta$ are indicated. B, Similar fractionation of type I thionins from barley endosperm. Variants $B\alpha$ and $B\beta$ are indicated. C, RP-HPLC fractionation of precipitate obtained between 35% and 55% of $(NH_4)_2SO_4$ from crude barley-leaf extract. Type II thionin variants BLa, BLb and BLc are indicated.

thionin solutions ($W\alpha_1$, $W\alpha_2$, $W\beta$, $B\alpha$ or $B\beta$) were sprayed on 7-day-old barley plants. The plants were grown for 7 days in 50 ml pots containing TKS1 soil, in a growth chamber (18°C, 12 h light, 50–60% relative humidity). Just before inoculation, the plants were transferred in the greenhose (20–22°C, 14 h light, 50–60% humidity) and kept there until the end of the experiment. The thionin solutions were sprayed on the upper leaf surface of the plants with a chromatography sprayer. One hour later (leaf surface still wet from the spray) or 2 days later (leaf surface completely dry), the plants were inoculated with *E. graminis* by shaking an infected plant full of spores above them and incubated in the greenhouse at 20°C. The disease severity was evaluated 7 days later by counting the number of lesions and by visual comparison of their sizes (mean of six replicates).

The test with *C. lagenarium* on cucumber was similarly carried out: the thionin solutions ($W\alpha_1$, $W\alpha_2$, $W\beta$, $B\alpha$ or $B\beta$) were sprayed on 1-week-old plants (cotyledon stage); 1 h or 2 days later, the plants were inoculated with *C. lagenarium* by spraying a spore suspension of 2×10^5 spores/ml on the cotyledons. The plants were incubated for 36 h in the dark with 100% R.H. and 20°C and then in a growth chamber with 14 h light, 40% R.H. and 20°C. The disease was evaluated 7 days after inoculation by visually estimating the number and size of the lesions (mean of six replicates).

3. Results

3.1. Purification of thionins

A crude preparation of type I thionins was obtained by precipitation with ethanol-HCl from the petroleum-ether extracts of mature endosperms from wheat and barley, followed by preparative electrophoresis, as previously described [12,13]. This procedure yields an essentially pure mixture of type I thionins both in wheat and in barley [12,13]. These preparations were subjected to HPLC, using H_2O/2-propanol gradients (Figs. 1A,B). Peaks corresponding to the different genetic variants were identified by amino acid analysis and comparison with the compositional data deduced from their known sequences [2]. In a typical run, 5 mg of the crude mixture were applied

and 1–1.5 mg of each variant were obtained. Apparent homogeneity of the purified proteins was ascertained by three different electrophoretic separations: SDS-PAGE; SGE, pH 3.2; and SGE, pH 8.3 (not shown). The three known variants of wheat type I thionin ($W\alpha_1$, $W\alpha_2$, $W\beta$) and the two variants of the same type from barley ($B\alpha$, $B\beta$) were thus purified.

Three type II variants (BLa, BLb, BLc) were purified from barley leaves by HPLC fractionation of a crude leaf thionin preparation (Fig. 1C). Fractions from the HPLC separation were subjected to SDS-PAGE and those giving single bands with about 5 kDa apparent molecular weights were further examined by SGE, pH 3.2 and SGE, pH 8.3, as well as by rechromatography in the same HPLC conditions, except that acetonitrile substituted 2-propanol (not shown). Three apparently homogeneous proteins were thus selected and their amino acid compositions were determined. In a typical run, 5 mg of crude thionin extract, obtained from 2 g of fresh tissue, were subjected to HPLC fractionation and 150–250 μg of each variant was obtained. Values of the compositional difference index [15] for the binary comparisons of the amino acid compositions of the three purified proteins (BLa, BLb and BLc) and those deduced from published nucleotide sequences for the leaf thionins [8] were in all cases well within those expected of homologous proteins. Furthermore, the amino acid composition of protein BLa was identical to that of thionin DG3 [8] and the com-

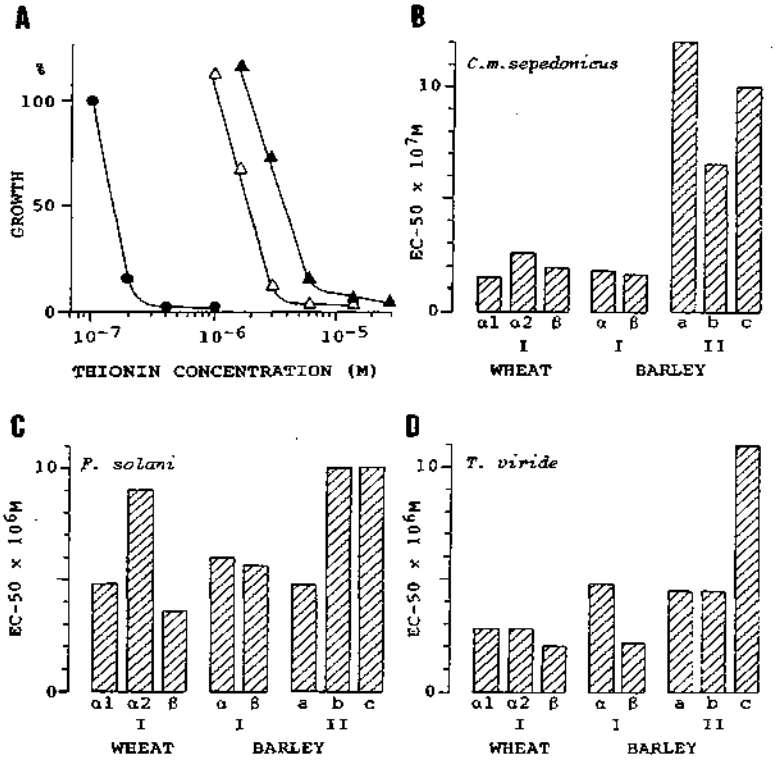

Fig. 2. In vitro activity of purified thionins of types I and II. A, Inhibition curves for the $W\alpha_1$ thionin against the bacterium *C. michiganensis* subsp. *sepedonicus* (●) and the fungi *F. solani* (▲) and *T. viride* (△). B-D, EC-50 values for the different thionins against the indicated microorganisms. Growth was determined in an ELISA plate reader as indicated in Materials and methods. Growth of each microorganism in medium without thionins was taken as 100%.

parisons between proteins BLb and BLc and thionins DB4 and DC4 gave values of the divergence index [15] that were similar to those obtained when comparing two purification batches of the same protein. To confirm their identification as thionins, the N-terminal sequence of BLa was determined up to the 25th residue and, as expected, was found to be identical to that deduced from the DG3 nucleotide sequence with no sequence heterogeneity.

3.2. Inhibitory activities of purified thionins

The eight purified thionins were tested against one bacterium, *C. michiganensis* subsp. *sepedonicus*, and two fungi, *F. solani* and *T. viride* (Fig. 2). These fungi, as well as most of the others subsequently tested showed moderate growth stimulation (10%–30%) at subinhibitory concentrations of thionins (Fig. 2A). The activities (EC-50 values) of the different thionins versus a given pathogen varied over a fivefold range, and the activity ranking of a given variant was different for the different pathogens (Fig. 2B–D). A survey of the in vitro activity of thionins against other bacterial and fungal pathogens is summarized in Table 2.

Thionins were also tested under conditions that mimicked in vivo situations: on potato leaves against *P. infestans*, on apple fruits, against *B. cinerea*, on barley leaves against *E. graminis* and on cucumber leaves against *C. lagenarium*. Only in the first case an inhibition of fungal growth was achieved (Table 3), whereas in the other cases, concentrations of up to 40 μM did not give any inhibition.

Table 3
In vivo activity of thionins against *Phytophthora infestans*

Product	Inhibition[a] (%) at		
	4 μM	40 μM	70 μM
Wα_1 thionin	0	64	ND
Wα_2 thionin	0	60	ND
Wβ thionin	0	100	ND
Bα thionin	40	100	ND
Bβ thionin	24	100	ND
Ridomil	ND	ND	86

[a]Mean of 6 replicates. 100%, no mycelium; 0%, growth as in water control. ND = not done.

4. Discussion

The HPLC procedures described here simplified the purification of type I thionins from wheat and barley and allowed the first isolation of the major type II thionins from barley leaves. The number of genes encoding leaf thionins in barley have been estimated by Southern blot analysis at 9–11 per haploid genome by Gausing [7] and at 50–100 by Bohlmann et al. [10]. Our finding of three main leaf thionins does not exclude the presence of lower concentrations of other type II thionins and suggests that most of the type II genes are either expressed at low levels or silenced (pseudogenes). Recovery of leaf thionins as abundant components of the soluble fraction is in agreement with previous observations indicating that at least 98% of leaf thionins are in the vacuoles and appear in the soluble fraction upon extraction [14].

As reviewed recently [2], the amino acid sequences of the thionin variants tested are highly conserved within types (>90% identical positions) and quite divergent across types (about 60% identical positions). Thionins of type I seemed to be more active against *C. michiganensis* subsp. *sepedonicus* than those of type II, whereas differences in activity against fungi among the different variants were only moderate. This relatively low variation of the inhibitory specificity among the different thionins present in a given species seems to exclude the specialization of each variant in the protection against a different pathogen.

While some bacterial strains tested were sensitive to thionins in the nanomolar range, the most sensitive fungi among those included in the present survey were inhibited by these proteins at micromolar concentrations. Other phytopathogenic bacteria previously tested [1] and other fungi included in this and in previous reports [10] required concentrations of one or two orders of magnitude higher for inhibition to occur. It will be of interest to investigate how readily resistance to thionins can be induced in pathogens.

We have previously shown that thionins alter

membrane permeability at concentrations that are equal or lower than those required to inhibit protein synthesis, which itself is more sensitive than that of RNA or DNA [21]. More recently, we have observed direct inactivation of certain enzymes by thionins through a mechanism that involves thiol-mediated protein–protein interactions [22]. This mechanism might be responsible for membrane leakage, as the effect can be abolished by reductants, such as dithiothreitol (unpublished). Differences in sensitivity among the different pathogens could reflect differences in the redox properties of the unknown target(s), but other possibilities, such as variation in the proportion of non-target binding sites, can not be excluded.

The fact that thionins were more effective than the fungicide Ridomil, on a molar basis, when used against *P. infestans* in a drop application assay on leaf discs from potato, suggests that the transgenic expression of thionin genes in potato might help to protect the plant against the pathogen. Concentrations of the same order (up to 50 μM) can be found in barley tissues and could be produced in the transgenic plants. The cases of *C. lagenarium* and *B. cinerea*, which are sensitive in vitro but are not controlled on the leaf or fruit surface, admit different explanations, including a possible higher proteolytic capacity of these fungi on the plant than in liquid medium, or inactivation of the thionins on certain plant surfaces.

5. Acknowledgements

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