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Expression of biologically active hordothionins in tobacco. Effects of pre- and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein expression and sorting

Dion E. A. Florack, Wim G. Dirkse, Bert Visser[1], Freek Heidekamp[2] and Willem J. Stiekema*
*DLO Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Department of Molecular Biology, P.O. Box 16, 6700 AA Wageningen, Netherlands (*author for correspondence); present addresses:*
[1] Ministry of Economic Affairs, Special Programme Biotechnology and Development Cooperation, The Hague, Netherlands; [2] Centre for Phytotechnology RUL/TNO, Leiden, Netherlands

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Abstract

Hordothionins (HTHs) are small anti-bacterial proteins present in barley endosperm which are processed from larger precursor proteins, consisting of an amino-terminal signal peptide (SP), the mature highly basic HTH and a carboxy-terminal acidic peptide (AP). Different HTH precursor proteins were expressed in tobacco to study the effects of the pre-sequences (SP) and pro-sequences (AP) on expression, processing, sorting and biological activity and hence the feasibility of engineering bacterial disease resistance into crops which lack these proteins. Maximum HTH expression levels of approximately 0.7% (11 μmol/kg) of total soluble protein in young tobacco leaves were obtained using a semi-synthetic gene construct encoding a complete chimaeric HTH precursor protein. Tenfold lower HTH expression levels (maximum 1.3 μmol/kg) were obtained using synthetic gene constructs without the AP-coding sequence and no expression was found in plants containing synthetic HTH gene constructs without SP-and AP-coding sequences. In both cases where expression was found, the precursors were apparently correctly processed, although the HTH produced in plants containing a construct without AP sequence appeared to be slightly modified. No effect on plant phenotype was observed. Localization studies indicated that the HTH was in identical fractions of plants expressing the two different precursors, albeit at a different ratio, and was not secreted into the intercellular spaces of leaves or culture medium by protoplasts. Our results indicated that the AP is not involved in sorting and suggested that it might facilitate transport through membranes. The *in vitro* toxicity of HTH isolated from transgenic tobacco plants expressing the two different precursor proteins for the bacterial plant pathogen *Clavibacter michiganensis* subsp. *michiganensis* appeared similar to that of the HTH purified from barley endosperm.

Introduction

Thionins are a family of low-molecular-weight (M_r ca. 5000) proteins that have been identified in various plant species (recently reviewed in [20, 21]). They can be divided into at least five different types, based on the number of amino acids, the net charge and the number of disul-

phide bonds present in the mature protein [7]. Best characterized at the molecular level are the type 1 thionins present in the endosperm of monocotyledons. Type 1 thionins have been isolated from wheat (purothionins; PTHs), barley (hordothionins; HTHs) and other monocots and were shown to be highly homologous. The PTHs appeared to consist of three proteins, designated α1-, α2- and β-PTH and the HTHs of two proteins, designated α- and β-HTH (reviewed in [21]). The type 1 thionins are composed of 45 amino acids, possess four disulphide bonds which are conserved in all the type 1 thionins known. They are synthesized as larger precursor proteins comprising three distinct domains: an amino-terminal signalpeptide (SP) involved in transition of the precursor into the lumen of the endoplasmic reticulum (ER), followed by the highly basic mature thionin and a carboxy-terminal acidic peptide (AP) with unknown function [23, 34, 35]. The type 1 PTHs and HTHs have been shown to exhibit anti-microbial properties against a number of bacteria, fungi and yeasts [2, 14, 17, 18, 22, 32, 33, 42].

The type 1 PTHs have also been shown to be toxic *in vitro* for a number of plant pathogenic bacteria at relatively low concentrations [14]. Toxicity differed slightly depending on the PTH used, α-PTH (a mixture of α1- and α2-PTH) or β-PTH for two of the bacteria tested [14]. This was confirmed for the type 1 HTHs which were shown to be toxic *in vitro* for several plant pathogenic bacteria causing serious damage in solanaceous crops [17]. This *in vitro* toxicity renders the genes encoding these proteins into potentially powerful tools for engineering bacterial disease resistance into plant species that lack these proteins [18, 21]. Prerequisites for such an application are high expression levels of the mature HTH, proper processing of precursor proteins and folding into the biologically active form and sorting of the protein to the compartment where the pathogen resides.

Recently, the constitutive expression in tobacco of a barley type 1 α-HTH gene accommodating two introns and controlled by the cauliflower mosaic virus (CaMV) 35S promoter, was shown to result in high HTH expression levels and enhanced resistance against two bacterial plant pathogens, *Pseudomonas syringae* pv. *tabaci* 153 and *P. syringae* pv. *syringae* [5]. In contrast, a wheat α1-PTH cDNA-derived gene, also controlled by the CaMV 35S promoter, was only poorly expressed in tobacco and did not result in resistance against these bacteria [5].

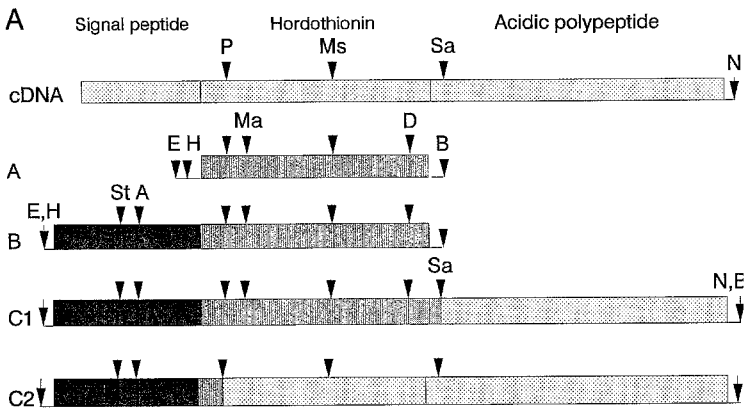
In this paper we report the introduction of seven synthetic and semi-synthetic gene constructs encoding different type 1 α- and β-HTH derived precursors into tobacco to study the effects of deletion of the N-terminal SP and/or C-terminal AP sequences on expression, processing and sorting and to study the biological activity of the mature HTH.

Materials and methods

Hordothionin plasmid constructions

The seven HTH gene constructs used in this work are represented in Fig. 1. Type A and B constructs were made chemically. The Type A constructs pAα and pAβ were designed based on the published sequences of two type 1 HTH cDNA

Fig. 1. Structures and sequences of the different HTH gene constructs used. A. Schematic drawing of the composition of a type 1 thionin cDNA and the type A, B, C1 and C2 gene constructs used in this work. Dotted boxes represent sequences of cDNA origin (pC263), grey and black boxes represent the chemically synthesized sequences coding for the mature protein and the signal peptide, respectively. The synthetic *Dde* I/*Sau* 96I linker adapter in type C1 constructs is indicated as a light-grey box. Vertical bars in the boxes indicate proteolytic cleavage sites. Restriction endonuclease cleavage sites are indicated by arrows (▼): A, *Ava* II; B, *Bam* HI; D, *Dde* I; E, *Eco* RI; H, *Hpa* I; Ma, *Mae* I; Ms, *Mst* I; N, *Nco* I; P, *Pst* I; Sa, *Sau* 96I; St, *Sty* I. B and C. Nucleotide and amino acid sequences of pAα, pAβ, pBα, pBβ, pC1α, pC1β and pC2α. For comparison, the corresponding sequence of a natural α-hordothionin gene, *Hth-1* [37], is shown on top. Translation start and stopcodons are printed in boldface. Flanking *Bam* HI, *Hpa* I and *Nco* I restriction sites are in italics and the optimized translation initiation region [29] is underlined. Sequences in common with the *Hth-1* gene are indicated by dots (.).



B.

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Hth-1                                ATGGGCCTCAAGGGTGTGATGGTGTGTTTACTTATACTGGGGTTGGTTCTCGAACAGGTGCAAGTAGAAGGC
pC2α      gttaacaATGGCACCCAGC.A.A.TA.T...A.....G.CA.T...G.T..C..C..T..A.....C..T.....A.............A
pC1ß      gttaacaATGGCACCCAGC.A.A.TA.T...A.....G.CA.T...G.T..C..C..T..A.....C..T.....T.............A
pC1α      gttaacaATGGCACCCAGC.A.A.TA.T...A.....G.CA.T...G.T..C..C..T..A.....C..T.....A.............A
pBß       gttaacaATGGCACCCAGC.A.A.TA.T...A.....G.CA.T...G.T..C..C..T..A.....C..T.....T.............A
pBα       gttaacaATGGCACCCAGC.A.A.TA.T...A.....G.CA.T...G.T..C..C..T..A.....C..T.....A.............A

Hth-1     ----------AAGAGTTGCTGCAGGAGCACCCTAGGAAGAAACTGCTACAACGTTTGCCGCGTCCGTGGTGCTCAGAAGTTATGCGCAGGGGTCT
pC2α      ----------.....................................................................................
pC1ß      ----------.....T..T..........T........T..........T..........A............AAT.CT.
pC1α      ----------.....T..T..........T........T..........T..........A.............A..T.
pBß       ----------.....T..T..........T........T..........T..........A............AAT.CT.
pBα       ----------.....T..T..........T........T..........T..........A.............A..T.
pAß       gttaacaATG.....T..T..........T........T..........T..........A............AAT.CT.
pAα       gttaacaATG.....T..T..........T........T..........T..........A.............A..T.

Hth-1     GTAGGTGTAAACTCACAAGTAGCGGAAAATGCCCTACAGGCTTCCCCAAA---TTGGCCCTTGTGTCAAACTCAGATGAACCAGACACCGTCAAG
pC2α      ...............................................---.....C.....................
pC1ß      .....G.ACT...G......T..A.T.....T..G---.....C.....................
pC1α      .....G.....A.....T..G---.....C.....................
pBß       .....G.ACT...G......T..A.T.....T..GTAGggatcc
pBα       .....G.....A.....T..GTAGggatcc
pAß       .....G.ACT...G......T..A.T.....T..GTAGggatcc
pAα       .....G.....A.....T..GTAGggatcc

Hth-1     TATTGGAACTTGGGGTGTAGGGCTTCCATGTGTGACTACATGGTCAACGCAGCTGCTGACGACGAAGAAATGAAACTCTATTTGGAAAATTGTGG
pC2α      ...............................................................................................
pC1ß      ...............................................................................................
pC1α      ...............................................................................................

Hth-1     TGATGCTTGTGTCAATTTCTGCAACGGTGATGCTGGGCTCACATCCCTTACTGCCTAATGAtgtgtatccatggtctgaga
pC2α      .....................................................................gggatcc
pC1ß      .....................................................................gggatcc
pC1α      .....................................................................gggatcc
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C.

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Hth-1              MVCLLILGLVLEQVQVEG-KSCCRSTLGRNCYNLCRVRGAQKLCAGVCRCKLTSSGKCPTGFPK
pC2α      MAPSKSIKSVVI.....H.....-.....
pC1ß      MAPSKSIKSVVI.....H.....-.....NA.....GL...SS...
pC1α      MAPSKSIKSVVI.....-.....
pBß       MAPSKSIKSVVI.....H.....-.....NA.....GL...SS...
pBα       MAPSKSIKSVVI.....-.....
pAß                                M.....NA.....GL...SS...
pAα                                M.....

Hth-1     LALVSNSDEPTVKYCNNLGCRAWMCDYMVNAAADDEEMKLYLENCGDACVNFCNGDAGLTSLTA
pC2α      ...........................................................
pC1ß      ...........................................................
pC1α      ...........................................................
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clones, pTH1 and pTH2 encoding α- and β-HTH respectively [23]. The Type B constructs pBα and pBβ were derived from the Type A constructs by the addition of the SP encoding sequence of a type 2 leaf thionin cDNA clone, pDB4 [1]. In these four synthetic genes the codon usage was altered in favour of use in solanaceous crops, as estimated from a comparison of compilated codon usages [46]. Some of the codons were also altered to create unique restriction sites at interval throughout the gene (Fig. 1). To simplify successive cloning experiments, an unique *Hpa* I site was created at the 5′ end as part of an optimized translation initiation region according to Lütcke *et al.* [29], and a *Bam* HI site at the 3′ end, directly following the stop codon (Fig. 1A and 1B). All synthetic genes were made by ligation of fragments derived after enzymatical conversion of 3′-end overlapping oligonucleotides as described previously [18]. The Type C1 constructs pC1α and pC1β were made by ligation of the *Sau* 96I/*Bam* HI fragment of pC263, a type 1 HTH cDNA clone (kindly provided by Dr A. Brandt, Carlsberg Research Center, Copenhagen) to pBα and pBβ, digested with *Hpa* I and *Dde* I using a synthetic *Dde* I/*Sau* 96I adapter. The Type C2 construct pC2α, was made by ligation of the *Hpa* I/*Pst* I fragment of construct pBβ to pC263, digested with *Pst* I and *Bam* HI. All gene constructs were initially cloned in Bluescribe pSK + (Stratagene, La Jolla, CA) and verified by sequencing using the dideoxy-mediated chain-termination method [40] on an Applied Biosystems 370A automated DNA sequencer. For expression in plants, all constructs were cloned as *Hpa* I/*Bam* HI fragments in the binary vector pCPO5 (Fig. 2). This binary vector was derived from pPCV708 [26] and contains between the left and right T-DNA borders three expression cassettes with multiple cloning sites. All HTH gene constructs were cloned between a modified CaMV 35S promoter containing a doubled enhancer sequence [25], and the nopaline synthase (NOS) terminator sequence. The recombinant binary vectors were conjugated into *Agrobacterium tumefaciens* GV3101 (pMP90) or GV3101 (pMP90RK) [27] by parental mating [44] using

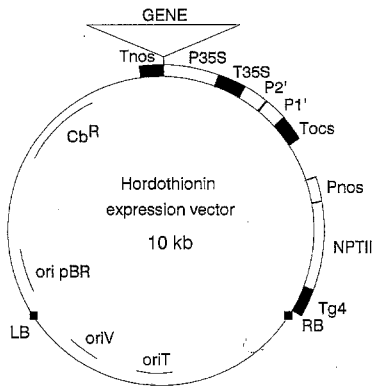

Fig. 2. Representation of the binary vector pCPO5 containing the different HTH gene constructs. RB, right T-DNA border sequence; Tg4, T-DNA gene 4 terminator; NPTII, neomycin phosphotransferase II gene, selectable kanamycin resistance marker; Pnos, nopaline synthase promoter; Tocs, octopine synthase terminator; P1′, T-DNA gene 1 promoter; p2′, T-DNA gene 2 promoter; T35S, CaMV 35S terminator; P35S, modified CaMV 35S promoter; Tnos, nopaline synthase terminator; Cb^R, bacterial ampicillin/carbenicillin resistance marker; ori pBR, pBR322 origin of replication; LB, left T-DNA border sequence; oriV, pRK2 origin of replication; oriT, pRK2 origin of conjugative transfer.

Escherichia coli HB101 (pRK2013) [13] as helper when appropriate.

Transformation of tobacco

Leaf discs of tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) were transformed by a modification of the method of Horsch *et al.* [24]. Shoots, regenerated in the presence of 100 mg/l kanamycin were rooted in MS medium [30], supplemented with 0.05 mg/l indole acetic acid (IAA) and 500 mg/l cefotaxim. After rooting, plantlets were transplanted to normal potting soil, transferred to the greenhouse and grown to maturity.

Hordothionin expression and processing analysis

Total RNA was isolated from young leaves (ca. 5 cm) of greenhouse-grown plants essentially as described [11]. 10 μg of total RNA from each plant was separated on a 2% agarose gel in the

presence of 6% (v/v) formaldehyde, transferred to membranes (GeneScreen Plus, New England Nuclear) for northern analysis [39] and hybridized with random primer ^{32}P-labelled DNA according to the manufacturer. The *Hpa* I/*Bam* HI fragments of Type A constructs were used as probes.

Proteins were also extracted from young leaves of greenhouse-grown plants. Ca. 100 mg of leaf tissue was ground in an Eppendorf tube under liquid nitrogen and incubated with 1 ml 50 mM H_2SO_4 for 1 h at 40 °C. After incubation, debris was pelleted by centrifugation and the supernatant was transferred to a fresh tube. Total protein was precipitated with ice-cold triochloroacetic acid (TCA) at a final concentration of 12% by overnight incubation at 4 °C. Precipitated proteins were collected by centrifugation, washed once with ice-cold absolute ethanol, lyophilized and dissolved in 200 μl 1% CH_3COOH. The amount of soluble protein was estimated by the method of Bradford [3].

For dot blot immuno-assays, 5 μg of soluble protein was blotted onto nitrocellulose membranes (BA 85, Schleicher & Schuell) using a dot blot apparatus (SRC 96 D, Schleicher & Schuell). Western blot analysis [43] was performed using a polyclonal antiserum raised in a rabbit against HTHs, coupled to bovine serum albumin (BSA) to increase antigenicity [9]. Alkaline phosphatase-labelled goat anti-rabbit IgG was from Sigma. HTH-expression levels were estimated by comparison of the results from samples of transgenic plants with the results from 50, 100, 150, 200 and 250 ng amounts of the purified HTHs from barley on dot blots. Transgenic plants were divided into 4 classes according to their HTH expression level: class 1, expression less than the detection level being 0.01% (<0.16 μmol/kg) of total soluble protein in young tobacco leaves (8 mg/g); class 2, expression between 0.01 and 0.03% (0.16–0.48 μmol/kg); class 3, expression between 0.03 and 0.1% (0.48–1.6 μmol/kg) and class 4, more than 0.1% (>1.6 μmol/kg) of total protein. Samples were analysed twice in independent experiments.

Proteins were analysed by SDS-tricine polyacrylamide gel electrophoresis (SDS-tricine-PAGE) according to Schägger and Jagow [41] and by acid PAGE as described previously [17]. Rainbow-coloured low-molecular-weight protein markers were from Amersham. Separated proteins were transferred to polyvinylidene difluoride (Immobilon-P, Millipore) or nitrocellulose (BA 85, Schleicher & Schuell) membranes in a Trans-Blot Cell (Bio-Rad) and were analysed by enhanced chemiluminescence western blotting (ECL, Amersham), using the HTH antiserum and horse radish peroxidase-labelled secondary antibody, according to the manufacturer.

Subcellular localization analysis

Intercellular washing fluids (IFs) were isolated from young leaves of selected transgenic plants containing the pAα, pBα and pC1α construct, and from control kanamycin resistant plants using water or a buffer containing 50 mM KH_2PO_4 and 100 mM NaCL (pH 7.2) to infiltrate the intercellular spaces [12]. 100 μl intercellular fluid of each plant was analysed by protein gel blotting as described above. Further analysis of the subcellular location of HTH was established by plant cell fractionation of the same, aseptically grown, plants essentially as described [10]. After incubation for 20 h in culture medium, protoplasts (2×10^6) were floated, separated from the medium which was kept as 'medium' fraction, and resuspended in 200 μl buffer, containing 50 mM Tris pH 7.5, 2 mM EDTA and 0.15 mg/ml PMSF. The protoplast membrane was disrupted by gentle passing through a yellow pipet tip and microsomes were separated from soluble cytoplasmic proteins by centrifugation at 14000 rpm for 10 min at 4 °C. The supernatant was kept as 'cytosol' fraction and the pellet was sonicated (5 μm amplitude, 20 s at 4 °C) in 200 μl of the buffer mentioned. Soluble microsomal proteins ('microsome' fraction) were separated from the membrane remnants ('membrane' fraction) by centrifugation at 14000 rpm for 10 min at 4 °C. All fractions were treated in 50 mM H_2SO_4 for 1 h at 40 °C and proteins were precipitated,

resuspended in 100 µl 1% CH_3COOH and analysed (25 µl of each fraction) by protein gel blotting for the presence of HTH as described above.

Analysis of biological activity

Protein extracts from ca. 200 g of leaf material from the selected plants described above were enriched for the HTH by selective water-ethanol treatment [15]. Proteins soluble in 70% ethanol were lyophilized and dissolved in a small volume of water. The amount of HTH present in these extracts was determined by the dot immunoassay. These extracts were tested for toxicity towards *Clavibacter michiganensis* subsp. *michiganensis* PD1386 (collection Dutch Plant Protection Service, Wageningen) in the micro-plate broth dilution assay as described previously [17]. As a control, a known amount of purified HTH-1 from barley endosperm was added to extracts from plants containing the pAα construct. An inoculum of 2×10^3 colony-forming units (CFUs) per ml was used and plates were incubated for 24 h at 27 °C. After incubation, CFUs were counted by plating serial ten-fold dilutions in trypticase soy agar (Becton-Dickinson) in 24-well Cluster[24] (Mark II, Costar) tissue culture plates.

Results

Hordothionin gene constructs and transformation

Gene constructs were prepared encoding different α- and β-HTH precursor proteins to investigate the necessity of a SP, the AP or both for expression, extracellular targeting and biological activity. Constructs designated Type A code for the mature protein domain only, Type B for the mature protein with a SP and Type C for the full-length precursor protein (Fig. 1A).

Type A and B constructs were synthesized chemically based on published type 1 HTH sequences. Codon usage in these constructs was altered in favour of use in solanaceous species, the crops we ultimately intend to engineer. In the type B constructs, the coding regions of the mature α- and β-HTH were preceded by the SP sequence derived from a type 2 barley leaf thionin [1]. This SP was favoured above the SP of the type 1 HTHs because it contains a stretch of positively charged amino acids at the NH_2 terminus, reported to enhance SP function [45]. Most of the SP sequence and the cleavage site remain unchanged, compared to the original SP sequence, due to the high homology (Fig. 1B and 1C). In all these synthetic genes the regions surrounding the ATG-codon were optimized for translation initiation according to Lütcke *et al.* [29]. Type C1 constructs were derived from the synthetic Type B constructs by addition of an AP-coding sequence, while the type C2 construct was derived from a type 1 HTH cDNA by addition of the SP-encoding region of the synthetic pBβ construct to the *Pst* I site of the cDNA (Fig. 1A). The nucleotide and amino acid sequences of all the constructs made are shown in Fig. 1B and 1C.

All constructs were cloned in the binary plant expression vector pCPO5 under control of a modified CaMV 35S promoter with a duplicated enhancer sequence and the Nos terminator sequence (Fig. 2) and introduced into plant cells. A large number of independent transgenic tobacco plants (52 plants with a type A, 113 with a Type B and 200 with a Type C construct) were obtained and grown to maturity in the greenhouse. As a control, the empty binary vector pCPO5 was introduced into tobacco (5 plants). All plants were phenotypically normal and most were self-fertile.

Hordothionin expression in tobacco

HTH-mRNA levels were estimated by Northern blot analysis of total RNA from 42 plants with a Type A, 30 plants with a Type B and 73 plants with a Type C gene construct. Hybridization with HTH-specific probes indicated that almost all the plants analysed expressed the gene. The level of HTH-mRNA varied depending on the type of construct used in the transformation event

(Fig. 3). Plants with a Type A construct gave only weak signals after Northern blot analysis, whereas the HTH-mRNA levels in plants with a Type B and C construct were higher. No differences were seen between plants containing a Type C1 or Type C2 construct.

HTH protein expression was studied of 48 plants with a Type A (pAα and pAβ), 80 plants with a Type B (pBα and pBβ) and 178 plants with a Type C (pC1α, pC1β and pC2α) gene construct by dot blot immuno-assays using the HTH antiserum. Proteins were extracted from ground leaf material by an extraction procedure previously used for the isolation of biologically active HTHs from barley endosperm [32]. On average, 100 μg of dilute sulphuric acid-extractable and acetic acid-soluble protein was extracted from 100 mg of leaf material (1 mg/g), which is 12.5% of the total amount of protein in these young leaves (ca. 8 mg/g fresh weight). 5 μg samples of these were blotted onto nitrocellulose membranes. Quantification was made possible by the inclusion of known amounts of purified HTHs from barley endosperm. Routinely, 50 to 100 ng HTH could be detected using this method. To allow comparison between dot blots, half of the samples on one blot were also included on the next blot and all samples were analysed twice. The protein could be detected in a large number of plants with a Type C and in approximately half of the plants with a Type B gene construct. In plants with a Type A gene construct, the HTH protein could not be detected in spite of the fact that almost all the plants expressed the synthetic HTH gene, as deduced from northern analyses (data not shown). Interestingly, most of the plants with a Type B construct showing HTH expression above the background level were transformed with the pBα construct, whereas only few plants containing the pBβ construct had detectable HTH

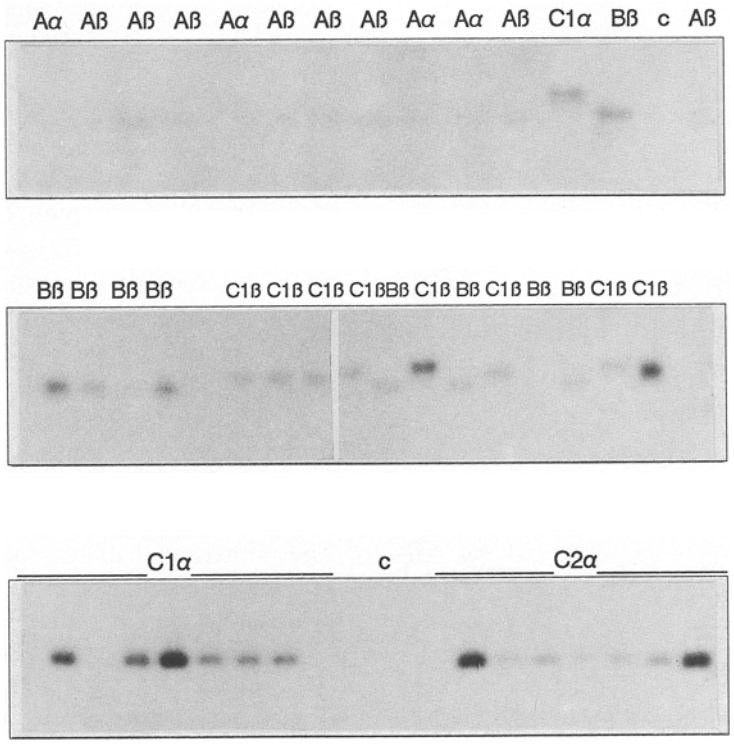

Fig. 3. Autoradiographs of northern blots containing total RNA of transgenic tobacco plants hybridized with HTH-specific probes. The construct type used for transformation in indicated on top of the lanes. The lane containing total RNA of a control kanamycin resistant plant is indicated by a 'c'.

protein. In young tobacco leaves of plants with a Type B construct (pBα), HTH levels up to ca. 0.1% (maximum 1.3 μmol/kg) of total protein were detected, and in plants with a Type C construct, HTH levels up to approximately 0.7% (11 μmol/kg) were detected. No differences were seen between the HTH protein levels of plants containing a Type C1 or Type C2 construct (data not shown). The HTH-mRNA level and the HTH-protein level in these plants was not correlated (data not shown), although the HTH-mRNA level was lower in plants with a Type A construct (Fig. 3) in which the HTH protein was not detectable. These transgenic plants were divided into four arbitrary expression classes which resulted in a distribution as shown in Fig. 4. These data indicate that the presence of a SP is essential for expression of the HTH, while the presence of an AP is essential for high expression.

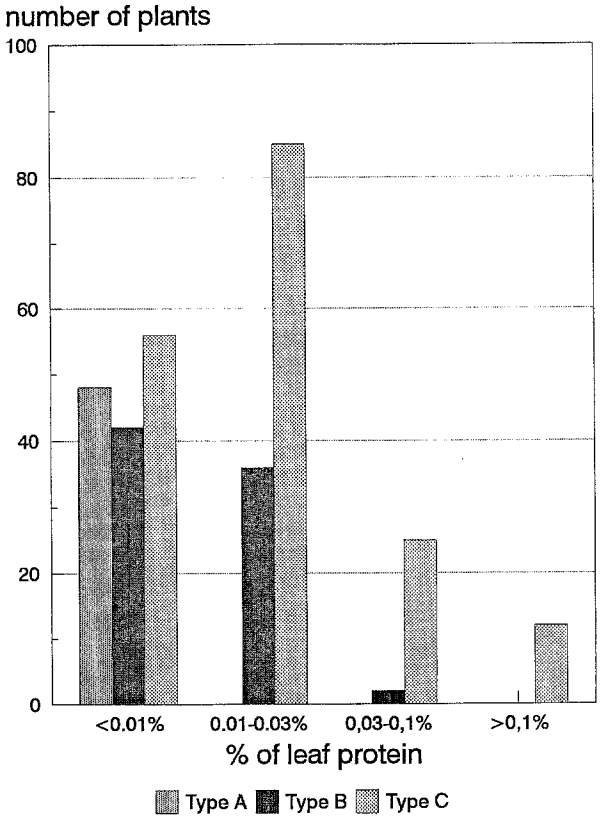

Fig. 4. Distribution of transgenic tobacco plants according to the HTH protein expression level. Expression of 48 plants containing a Type A, 80 plants containing a Type B and 178 plants containing a Type C gene construct were analyzed by dot blot immuno-assays and the expression levels were divided into four arbitrary expression classes. Class 1, expression less than the detection level being 0.01% (<0.16 μmol/kg) of total soluble protein; class 2, HTH expression between 0.01 and 0.03% (0.16–0.48 μmol/kg); class 3, HTH expression between 0.03 and 0.1% (0.48–1.6 μmol/kg) and class 4, expression higher than 0.1% (>1.6 μmol/kg) of total protein. Samples were analyzed twice in independent experiments. The numbers of plants (y-axis) per type of construct present in each expression class (x-axis) are indicated.

Hordothionin precursor processing

One plant each containing the pAα, pBα and pC1α construct were chosen for further analysis of processing, subcellular localization and biological activity of the HTH. The plants containing the pBα (ANF901710; HTH level 0.64 μmol/kg) and the pC1α (ANF902932; HTH level 2.2 μmol/kg) gene construct were chosen on the basis of a moderate respectively high HTH-protein expression, while the selected plant containing the pAα construct (ANF903401) was chosen on the basis of moderate HTH-mRNA expression, because no HTH was detected in this plant. A control kanamycin-resistant plant (ANF900701) was also included. The immunoblot of the protein extract from ANF902932 (pC1α) separated on a SDS-tricine polyacrylamide gel (Fig. 5A, lane 3) showed a single band which comigrated with HTH-1 (Fig. 5A, lane 5) purified from barley endosperm and which probably represents α-HTH [17]. The apparent molecular weight was 5 kDa, which is in agreement with that of the mature α-HTH encoded by these constructs as deduced from the amino acid sequence ([23]; see Fig. 1C). The immunoblot of the same extract separated on an acid polyacrylamide gel (Fig. 5B, lane 3) also showed one band comigrating with the biologically active HTH from barley endosperm (Fig. 5B, lane 5). Both immunoblots of the extract from ANF901710 (pBα) also showed a single band, but the protein recognized by the antibody migrated slightly slower on both gels (Fig. 5A and 5B, lane 2). On the contrary, the immunoblots of

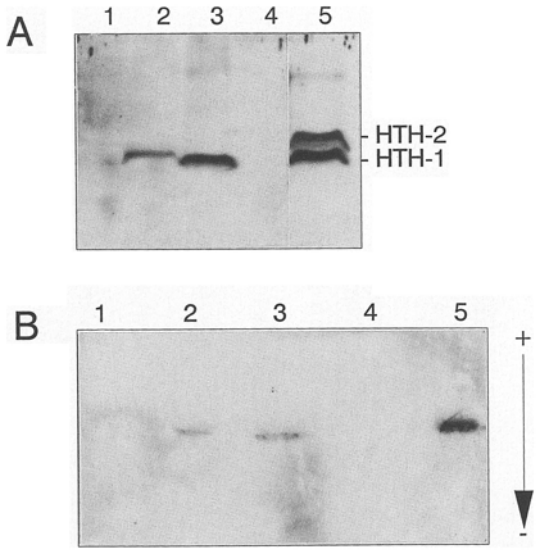

Fig. 5. Immunoblots of leaf proteins from tobacco plants transformed with the pAα, pBα and pC1α constructs after incubation with the HTH antibody. Lane 1, ANF900701, kanamycin-resistant control plant; lane 2, ANF901710 (pBα); lane 3, ANF902932 (pC1α); lane 4, ANF903401 (pAα) and lane 5, purified HTH from barley endosperm. A. Proteins were separated by SDS tricine PAGE. The position of HTH-1 and HTH-2 [17] from barley endosperm are indicated at the right. B. The same proteins separated by acid PAGE. The migration direction (+ to −) is indicated at the right.

protein extracts from ANF903401 (pAα; Fig. 5A and 5B, lane 4) and from the control kanamycin-resistant plant ANF900701 (Fig. 5A and 5B, lane 1) showed no bands. From these experiments it appears that the precursor proteins are correctly processed at the junctions of the SP and mature HTH and at the junction of the mature HTH and the AP. The different migration of the HTH produced in the pBα plant on both gels might be explained by a minor modification of the mature α-HTH domain.

Localization of HTH in transgenic tobacco

The same plants analysed for HTH processing were used to study the subcellular location of the mature HTH. Western blot analysis of IFs of these plants revealed that HTH was not secreted into the intercellular spaces (data not shown). This prompted us to investigate in which compartment HTH accumulated. Protoplasts were prepared of aseptically grown plants and fractionated into a 'medium', 'cytosol', 'microsome' and 'membrane' fraction. Immunoblots of the proteins from the four fractions of ANF902932 (pC1α) separated on SDS-tricine polyacrylamide gels (Fig. 6A and 6B, lanes 5 and 10) indicated that HTH was present in the 'cytosol', 'microsome' and 'membrane' fraction at an

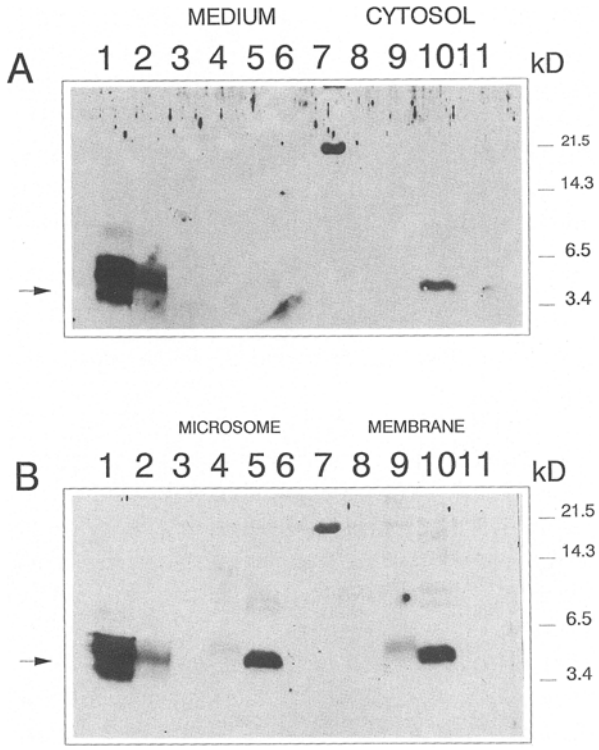

Fig. 6. Immunoblots of the different fractions from tobacco protoplasts of plants transformed with the pAα, pBα and pC1α construct and control plants after incubation with the HTH antibody. All fractions were treated with dilute sulphuric acid. Lanes 1 and 2, 100 and 50 ng HTH respectively; lanes 3 and 8, ANF900701, control kanamycin-resistant plant; lanes 4 and 9, ANF901710 (pBα); lanes 5 and 10, ANF902932 (pC1α); lanes 6 and 11, ANF903401 (pAα) and lane 7, low-molecular-weight size markers. A. Immunoblot of medium- (MEDIUM) and cytoplasm-enriched (CYTOSOL) fractions. B. Immunoblot of the contents of endomembrane compartment- (MICROSOME) and membrane remainder-enriched (MEMBRANE) fractions. Molecular weight size markers are indicated at the right and the position of the HTH is indicated by an arrow at the left.

approximate ratio 1:5:5 and comigrated with the HTH from barley endosperm (Fig. 6A and 6B, lanes 1 and 2). The apparent molecular weight was 5 kDa, which is in agreement with the results from the processing analysis (Fig. 5). Immunoblots of the four fractions of ANF901710 (pBα) indicated that HTH was present in the 'microsome' and 'membrane' fraction at an approximate ratio of 1:5 (Fig. 6B, lanes 4 and 9) and migrated slightly slower, confirming our previous findings. As expected, the immunoblots containing the fractions from the two control plants showed no bands (Fig. 6A and 6B, lanes 3, 6, 8 and 11). The faint bands of higher molecular weight in the 'microsome' and 'membrane' fractions of ANF902932 (Fig. 6B, lanes 5 and 10), might be HTH precursors derived by processing of the AP, since these were not detected in the fractions from ANF901710 (pBα) which lacked the C-terminal AP, and in control plants.

Biological activity of hordothionins from transgenic plants

The experiments described above indicated that the different HTH precursor proteins were correctly processed and expressed up to high levels in tobacco plants, depending on the construct used for transformation. Extracts from these transgenic plants were enriched for HTHs by selective water-ethanol treatment and tested for *in vitro* toxicity towards *C. michiganensis* subsp. *michiganensis* in micro-plates. This bacterial plant pathogen was previously shown to be susceptible to HTHs [17]. Serial two-fold dilutions of the partially purified extracts were inoculated and after incubation and plating, bacteria were counted. The results indicated that toxicity of the extracts from plants transformed with the pBα and pC1α constructs, increased with increasing amounts of protein, and toxicity was similar to that of the purified HTH-1 from barley endosperm (Fig. 7). In contrast, extracts from plants containing the pAα construct were not toxic at similar protein levels.

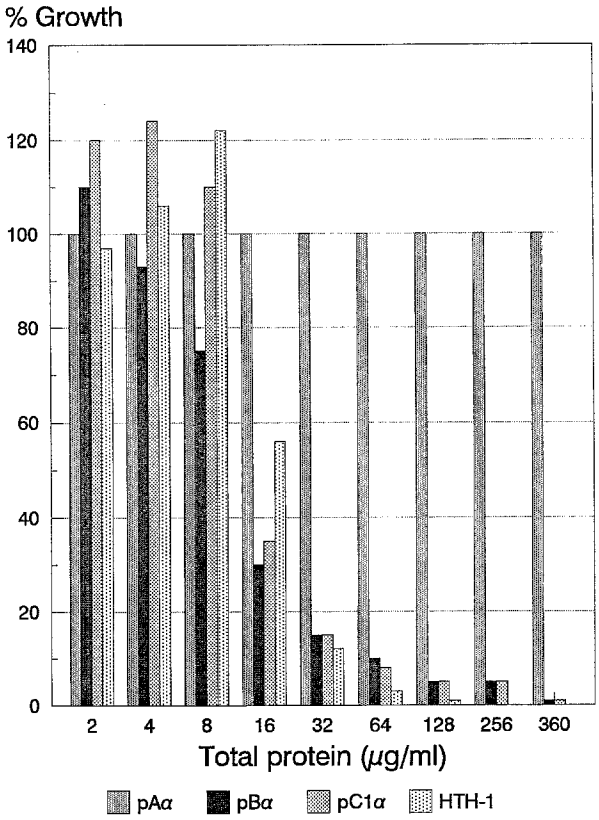

Fig. 7. In vitro growth of the bacterial plant pathogen *Clavibacter michiganensis* subsp. *michiganensis* PD1386 in serial twofold dilutions of partially purified extracts from transgenic plants. Growth in extracts from plants containing the pBα construct (ANF901710) and the pC1α construct (ANF902932) was compared to growth in extracts from plants harbouring the pAα construct (ANF903401), for which growth was taken as 100% at each dilution tested. As a control, purified HTH-1 from barley was mixed with extract of ANF903401, and growth was compared to growth in extract of ANF903401. This experiment is one of three performed which gave similar results. The concentrations of HTH present in each of the most concentrated samples (360 μg/ml) were 9.6 μM for HTH-1, 5 μM for pC1α extract and 2.5 μM for pBα extract. The bacterial inoculum was 2.3×10^3 CFU/ml and plates were incubated for 24 h at 27 °C before plating.

Discussion

The aim of our research was to investigate the feasibility of using the thionins from barley endosperm for engineering bacterial disease resistance into plants. To this end, expression of the mature HTH, processing of different precursor proteins, subcellular targeting and biological

activity were studied in tobacco using three different types of HTH gene constructs (Fig. 1). Our results clearly indicate that HTHs can only be expressed when fused to a SP sequence. SP sequences mediate the transition of proteins into the endoplasmic reticulum (ER; reviewed in [8]) where conditions for disulphide bond formation are favourable and enzymes like protein disulphide isomerase are present which catalyse the formation of these bonds [19, 38]. Disulphide bond formation is a prerequisite for the correct folding and highly stable conformation of HTHs, which might explain our inability to detect HTH in extracts of plants transformed with Type A gene constructs which lack a SP sequence. The HTH produced in these plants probably cannot form disulphide bonds in the reducing environment of the cytosol [19] which, in addition, does not contain the enzyme protein disulphide isomerase [38], thereby resulting in an unstable protein. We also showed that HTH accumulates to at least tenfold higher levels in plants containing Type C gene constructs with the AP sequence as compared to plants containing Type B gene constructs without the AP sequence (Fig. 4). One transgenic plant each harboring the constructs coding for the different α-HTH precursor proteins were chosen for further analysis because the HTH was easily detectable in these plants, whereas the HTH was hardly detectable in plants containing the pB β construct. Protein gel blotting revealed that the HTH had a molecular mass of ca. 5 kDa, which indicates correct processing of both precursors (Fig. 5). It also showed that the HTH produced in tobacco by the pBα construct migrated slightly slower on both gel types compared to the HTH produced in the plant with the pC1α construct and the barley endosperm HTH (Fig. 5). This might be the result of a modification of the mature α-HTH in plants with the pBα construct, which might be prevented in plants with the pC1α construct as the result of a close interaction of the AP with the mature α-HTH (see below). In fact, there is a potential protein kinase C phosphorylatable serine residue at position 36 in the mature α-HTH domain [47]. This serine residue is flanked by a number of basic residues (see Fig. 1C), which enhance the kinetic parameters for protein kinase C phosphorylation [47]. Phosphorylation of a serine residue has only a minor effect on the molecular weight of a protein, but could have a major effect on the overall charge. In our case the α-HTH produced by the plant with the pBα construct migrated slightly slower on both gels (Fig. 5A and 5B), which might be explained by the fact that the acid polyacrylamide gel was run at pH 4.3 at which point the addition of two negatively charged groups only has a minor effect on the overall charge of the α-HTH compared to the ten positively charged lysine and arginine residues. Experiments to unravel the exact kind of the modification remain to be performed.

HTH was not secreted into the intercellular spaces of leaves and also not found in the 'medium' fraction of protoplasts from these plants (Fig. 6A). It was found in the 'microsome' and 'membrane' fractions of plants containing the pBα or pC1α construct, suggesting that the AP is not involved in sorting and that the signal for intracellular retention must be in the mature protein domain. The presence of HTH in the membrane fraction is in agreement with sucrose gradient centrifugation experiments performed on barley endosperm where the HTH was found to cosediment with the ER and membranes of the protein bodies [4, 34] and immunogold labelling performed on barley leaves which indicated that the (type 2) thionin was present in the vacuoles [36] and cell wall [2]. These type 2 thionins were also not found in the intracellular spaces of barley leaves [16]. The localization experiments also showed that the HTH produced by the plant with the pBα construct had a slightly different mobility on SDS-tricine polyacrylamide gels, which is in agreement with our results obtained in the processing analysis experiments. The presence of HTH in the cytosol of protoplasts from the pC1α plant, which was selected for high expression (ca. 0.25% of total protein in young tobacco leaves), most likely is caused by disruption of the membranes of the microsomes (mainly vacuoles in plants) during preparation of the cytoplasm-enriched fraction, although leakage from the

microsomes caused by the intrinsic pore-forming ability of HTHs [6, 31, 33] cannot be excluded. The largest amount of HTH from the plant with the pBα construct was in the 'membrane' fraction (Fig. 6B, lane 9 versus lane 4) whereas in plants expressing the pC1α construct the ratio was equal for 'microsome' and 'membrane' fractions (Fig. 6B, lanes 5 and 10), suggesting that the HTH integrated in some membrane (maybe the ER) in the absence of an AP.

In view of these results, two possible functions could be ascribed to the AP. The first might be that the negatively charged AP functions as an intramolecular chaperone by stimulating the correct folding of the extremely positively charged mature HTH, resulting in higher protein expression levels. This is in analogy with the interaction of the negatively charged nucleoplasmin with the positively charged histones in *Xenopus* eggs, essential for the assembly of nucleosomes from DNA and histones, described by Laskey *et al.* [28], who first used the term 'molecular chaperone' to describe this function of the nucleoplasmin. A second possible function of the AP might be a role in neutralizing the extremely positive charge of the mature HTH during transit through the secretory pathway, thereby preventing illegitimate interactions with other components of the pathway. Toxicity of thionins is caused by pore formation in biological membranes most likely after binding to its polar lipids and this might hamper direct transport of mature thionin through the ER. This proposed role of the AP is favoured by our findings that only low amounts of HTH are found in plants with a Type B construct and that the HTH in these plants is mainly found in the membrane fraction. Our findings that the HTH produced in plants with a pBα construct might be modified, further strengthens a possible close interaction of the AP with the mature HTH during transition through the secretory pathway. In case of protein kinase C phosphorylation the presence of an AP sequence, as in the pC1α construct, would then result in inaccessibility of the serine residue at position 36 of the mature α-HTH domain to phosphorylation.

We have shown that the barley endosperm thionins can be expressed by a Type B construct and accumulate up to high levels in transgenic tobacco plants with a Type C gene construct (Figs. 4, 5 and 6). Carmona *et al.* [5] recently showed that a cDNA-derived gene encoding α1-PTH, under control of a CaMV 35S promoter, was only poorly expressed in tobacco with PTH levels less than 0.2 μmol/kg. In contrast, expression of our Type C HTH gene constructs, which resemble this PTH gene in structure, resulted in 100-fold higher expression of the mature HTH. This might be explained by the differences between these constructs, encompassing a doubled CaMV 35S promoter, the origin of the SP sequence, the origin of the thionin (puro- versus hordothionin), the codon usage and the optimized translation initiation region, or by differences in the calculation of protein levels. The HTH expression levels found in transgenic tobacco harbouring a genomic HTH gene construct under control of a CaMV 35S promoter [5], were similar to those found in our transgenic plants harbouring a Type C construct.

The most important prerequisite for application of HTHs in biotechnology is the biological activity of these proteins when produced in transgenic plant cells. Expression of a genomic DNA encoding α-HTH in tobacco, resulted in high HTH expression levels and enhanced resistance to *P. syringae* pv. *tabaci* 153 and *P. syringae* pv. *syringae* [5]. Recently, we demonstrated that the type 1 HTHs from barley are toxic for *C. michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker in tomato, *C. michiganensis* subsp. *sepedonicus*, the causal agent of ring rot in potato and *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of a spot disease in tomato and pepper [17], bacteria that are not pathogenic for tobacco. Bacteria that are pathogenic for tobacco and were available to us, respectively *P. solanacearum* (biovar 1, race 1) UW213 (= PD1456), *P. syringae* pv. *tabaci* NCPPB1408 (= PD1616) and *P. syringae* pv. *tabaci* NCPPB1427 (= PD1617) were not sensitive for HTHs in contrast to *P. syringae* pv. *tabaci* 153 used by Carmona *et al.* [5]. This might be explained by differences in bacterial strains. There-

fore our transgenic tobacco plants were not further evaluated for resistance and another approach was chosen to determine the biological activity. *In vitro* assays clearly indicated that the HTHs from the transgenic tobacco plants were biologically active and inhibited the growth of *C. michiganensis* subsp. *michiganensis*. Experiments to determine the level of resistance of transgenic tomato plants, harbouring a Type C HTH gene construct giving highest expression in tobacco, by infection with *C. michiganensis* subsp. *michiganensis* and *X. campestris* pv. *vesicatoria* and transgenic potato plants by infection with *C. michiganensis* subsp. *sepedonicus* remain to be performed.

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References

1. Bohlmann H, Apel K: Isolation and characterization of cDNAs coding for leaf-specific thionins closely related to the endosperm-specific hordothionin of barley (*Hordeum vulgare* L.). Mol Gen Genet 207: 446–454 (1987).
2. Bohlmann H, Clausen S, Behnke S, Giese H, Hiller C, Reimann-Philipp U, Schrader G, Barkholt V, Apel K: Leaf-specific thionins of barley – a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defense mechanism of plants. EMBO J 7: 1559–1565 (1988).
3. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254 (1976).
4. Carbonero P, García-Olmedo F, Hernández-Lucas C: External association of hordothionins with protein bodies in mature barley. J Agric Food Chem 28: 399–402 (1980).
5. Carmona MJ, Molina A, Fernández JA, López-Fando JJ, García-Olmedo F: Expression of the α-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. Plant J 3: 457–462 (1993).
6. Carrasco L, Vásquez D, Hernández-Lucas C, Carbonero P, García-Olmedo F: Thionins, plant peptides that modify membrane permeability in cultured mammalian cells. Eur J Biochem 116: 185–189 (1980).
7. Castagnaro A, Maraña C, Carbonero P, García-Olmedo F: Extreme divergence of a novel wheat thionin generated by a mutational burst specifically affecting the mature protein domain of the precursor. J Mol Biol 224: 1003–1009 (1992).
8. Chrispeels MJ: Sorting of protein in the secretory system. Annu Rev Plant Physiol Plant Mol Biol 42: 21–53 (1991).
9. Deen C, Claassen E, Gerritse K, Zegers ND, Boersma WJA: A novel carbodiimide coupling method for synthetic peptides: enhanced anti-peptide antibody responses. J Immunol Meth 129: 119–125 (1990).
10. Denecke J, Botterman J, Deblaere R: Protein secretion in plant cells can occur via a default pathway. Plant Cell 2: 51–59 (1990).
11. De Vries S, Hoge H, Bisseling T: Isolation of total and polysomal RNA from plant tissues. In: Gelvin B, Schilperoort RA, Verma DPS (eds) Plant Molecular Biology Manual, pp. B6/1–B6/13. Kluwer Academic Publishers, Dordrecht (1991).
12. De Wit PJGM, Spikman G: Evidence for the occurrence of race and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interactions of *Cladosporium fulvum* and tomato. Physiol Plant Path 21: 1–11 (1982).
13. Ditta G, Stanfield S, Corbiu D, Helinski D: Broad host range DNA cloning system for gram-negative bacteria: construction of a genebank of *Rhizobium meliloti*. Proc Natl Acad Sci USA 77: 7347–7351 (1980).
14. Fernandez de Caleya R, Gonzalez-Pascual B, García-Olmedo F, Carbonero P: Susceptibility of phytopathogenic bacteria to wheat purothionins *in vitro*. Appl Microbiol 23: 998–1000 (1972).
15. Fisher N, Redman DG, Elton GAH: Fractionation and characterization of purothionin. Cereal Chem 45: 48–57 (1968).
16. Fisher R, Behnke S, Apel K: The effect of chemical stress on the polypeptide composition of the intercellular fluid of barley leaves. Planta 178: 61–68 (1989).
17. Florack DEA, Visser B, de Vries PhM, van Vuurde JWL, Stiekema WJ: Analysis of the toxicity of purothionins and hordothionins for plant pathogenic bacteria. Neth J Plant Path, in press (1993).
18. Florack DEA, Visser L, Van Vloten-Doting L, Heidekamp F, Stiekema WJ: Synthetic hordothionin genes as tools for bacterial disease resistance breeding. In: Dekkers JJ, van der Plas HC, Vuijk DH (eds), Agricultural Bio-

technology in Focus in the Netherlands, pp. 39–46. Pudoc, Wageningen (1989).
19. Freedman RB: Protein disulphide isomerase: multiple roles in the modification of nascent secretory proteins. Cell 57: 1069–1072 (1989).
20. García-Olmedo F, Carmona MJ, Lopez-Fando JJ, Fernandez JA, Castagnaro A, Molina A, Hernández-Lucas C, Carbonero P: Characterization and analysis of thionin genes. In: Boller T, Meins F (eds) Gene Involved in Plant Defense, pp. 283–302. Springer-Verlag, Wien (1992).
21. García-Olmedo F, Rodriguez-Palenzuela P, Hernández-Lucas C, Ponz F, Maraña C, Carmona MJ, Lopez-Fando J, Fernandez JA, Carbonero P: The thionins: a protein family that includes purothionins, viscotoxins and crambins. Oxford Surv Plant Mol Cell Biol 6: 31–60 (1989).
22. Hernández-Lucas C, Fernandez de Caleya R, Carbonero P: Inhibition of brewer's yeasts by wheat purothionins. Appl Microbiol 28: 165–168 (1974).
23. Hernández-Lucas C, Royo J, Paz-Ares J, Ponz F, García-Olmedo F, Carbonero P: Polyadenylation site heterogeneity in mRNA encoding the precursor of the barley toxin α-hordothionin. FEBS Lett 200: 103–105 (1986).
24. Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT: A simple and general method for transferring genes into plants. Science 227: 1229–1231 (1985).
25. Kay R, Chan A, Daley M, McPherson J: Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236: 1299–1302 (1987).
26. Koncz Cs, Mayerhofer R, Koncz-Kalman Z, Nawrath C, Reiss B, Redei GP, Schell J: Isolation of a gene encoding a novel chloroplast protein by T-DNA tagging in *Arabidopsis thaliana*. EMBO J 9: 1337–1346 (1990).
27. Koncz Cs, Schell J: The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet 204: 383–396 (1986).
28. Laskey RA, Honda BM, Mills AD, Finch JT: Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. Nature 275: 416–420 (1978).
29. Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA: Selection of AUG initiation codons differs in plants and animals. EMBO J 6: 43–48 (1987).
30. Murashige T, Skoog F: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497 (1962).
31. Oka T, Murata Y, Nakanishi T, Yoshizumi H, Hayashida H, Ohtsuki Y, Toyoshima K, Hakura A: Similarity, in molecular structure and function, between the plant toxin purothionin and the mammalian pore-forming proteins. Mol Biol Evol 9: 707–715 (1992).
32. Okada T, Yoshizumi H: A lethal toxic substance for brewing yeast in wheat and barley. Part II. Isolation and some properties of toxic principle. Agric Biol Chem 34: 1089–1094 (1970).
33. Okada T, Yoshizumi H: The mode of action of toxic protein in wheat and barley on brewing yeast. Agric Biol Chem 37: 2289–2294 (1973).
34. Ponz F, Paz-Ares J, Hernández-Lucas C, Carbonero P, García-Olmedo F: Synthesis and processing of thionin precursors in developing endosperm from barley (*Hordeum vulgare* L.). EMBO J 2: 1035–1040 (1983).
35. Ponz F, Paz-Ares J, Hernández-Lucas C, García-Olmedo F, Carbonero P: Cloning and nucleotide sequence of a cDNA encoding the precursor of the barley toxin α-hordothionin. Eur J Biochem 156: 131–135 (1986).
36. Reimann-Philipp U, Schrader G, Martinoia E, Barkholt V, Apel K: Intracellular thionins of barley. A second group of leaf thionins closely related to but distinct from cell wall-bound thionins. J Biol Chem 264: 8978–8984 (1989).
37. Rodriguez-Palenzuela P, Pintor-Toro JA, Carbonero P, García-Olmedo F: Nucleotide sequence and endosperm-specific expression of the structural gene for the toxin α-hordothionin in barley (*Hordeum vulgare* L.). Gene 70: 271–281 (1988).
38. Rothman JE: Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell 59: 591–601 (1989).
39. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
40. Sanger F, Nicklen S, Coulson AR: DNA sequencing with the chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).
41. Schägger H, von Jagow G: Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166: 368–379 (1987).
42. Stuart LS, Harris TH: Bactericidal and fungicidal properties of a crystalline protein isolated from unbleached wheat flour. Cereal Chem 19: 288–300 (1942).
43. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354 (1979).
44. van Larebeke N, Engler G, Holster M, van den Elsacker S, Zaenen I, Schilperoort RA, Schell J: Large plasmid in *Agrobacterium tumefaciens* essential for crown gall inducing activity. Nature 252: 169–170 (1974).
45. von Heijne G: Analysis of the distribution of charged residues in the N-terminal region of signal sequences: implications for protein export in prokaryotic and eukaryotic cells. EMBO J 3: 2315–2318 (1984).
46. Wada K-N, Aota S-I, Tsuchiya R, Ishibashi R, Gojobori T, Ikemura T: Codon usages tabulated from the GenBank genetic sequence data. Nucl Acids Res 18: 2367–2411 (1990).
47. Woodget JR, Gould KL, Hunter T: Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. Eur J Biochem 161: 177–184 (1986).