APPLICATION OF HORDOTHIONINS AND CECROPIN B FOR ENGINEERING BACTERIAL DISEASE RESISTANCE INTO PLANTS

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 21 september 1994
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

This work was carried out at CPRO-DLO in the framework of the research programs "Protection of *Solanaceae* against phytopathogenic microorganisms by the introduction of synthetic hordothionin genes", supported by the Dutch Programme Committee Agricultural Biotechnology (PcLB), and "Protection of willow against the watermark disease by the expression of antibacterial proteins", supported by the arboriculture companies Boomkwekerij Udenhout B.V. and Ton van den Oever B.V., and the Dutch Programmatic Business orientated Technology Stimulation Fund (PBTS).

Cover design: Marieke

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Florack, Dion

Application of hordothionins and cecropin B for engineering bacterial disease resistance into plants / Dion Florack. - [S.l. : s.n.]. - Ill.
Proefschrift Wageningen. - Met lit. opg., reg. - Met samenvatting in het Nederlands.
ISBN 90-5485-280-1 geb.
Trefw.: biotechnologie / genetische modificatie / resistentieveredeling.

Stellingen

1. De classificatie van een aantal kleine basische eiwitten uit gerst, tarwe en tabak als γ-thioninen, is onterecht en getuigt van onbegrip van de nomenclatuur van deze eiwitten.

   Mendez *et al.* (1990) Eur J Biochem 194: 533-539.
   Colilla *et al.* (1990) FEBS Lett 270: 191-194.
   Gu *et al.* (1992) Mol Gen Genet 234: 89-96.

2. In de gentechnologie kan het vaak voordelig zijn om synthetische genen te gebruiken in plaats van uit biologisch materiaal geisoleerde genen.

   Gröger *et al.* (1988) Nucl Acids Res 16: 7763-7771.

3. Het Vaticaan heeft minder problemen met synthetische, dan met uit biologisch materiaal afkomstige genen, wat betreft het toepassen van genen.

   Mgr. E. Sgreccia, Secretaris van de Pauselijke Raad voor het Gezin.

4. De verminderde groei van *Pseudomonas syringae* pv. *tabaci* in bladeren van transgene tabak met hoge hordothionine expressie, zoals die wordt gerapporteerd door Carmona *et al.* (1993), valt moeilijk te rijmen met het feit dat hordothionine niet gesecreteerd wordt en niet in de intercellulairen voorkomt.

   Carmona *et al.* (1993) The Plant J 3: 457-462.
   Dit proefschrift.

5. Het zure polypeptide domein van de hordothionine precursor vergemakkelijkt het transport van de precursor door het endoplasmatisch reticulum naar de vacuole.

   Dit proefschrift.

6. De grote gevoeligheid van cecropine B voor proteases die aanwezig zijn in planten, belemmert een succesvolle toepassing van dit soort eiwitten voor het verkrijgen van bacterieresistente planten.

   Dit proefschrift.

7. Het toestaan van het gebruik van aluminium-verbindingen, die op de zwarte lijst van milieuschadelijke stoffen staan, in houdbaarheidsmiddelen voor snijbloemen, getuigt van tweesporen-beleid van de ministeries van VROM en LNV.

   Meerjarenplan Gewasbescherming; Rapportage Werkgroep Bloemisterij, Ministerie van LNV

Stellingen behorende bij het proefschrift: "Application of hordothionins and cecropin B for engineering bacterial disease resistance into plants", te verdedigen op woensdag 21 september 1994 des namiddags om vier uur door D.E.A. Florack.

en een klein beetje voor Marieke, Marlou, Dion jr. en Floran

CONTENTS

General introduction and outline of this thesis

Bacterial diseases can cause a severe decrease of yield in certain crops. The losses of the worldwide potato production can be as high as 25% due to bacterial infection [Sawyer, 1984]. The same is true for tomato and tobacco in certain parts of the world. Breeding for bacterial disease resistance therefore is of the utmost importance. The plant pathogenic bacteria causing most serious damage belong to the genera *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas* and *Xanthomonas*. For each of these genera, a number of subspecies (subsp.), pathovars (pv.), races or types are known, each on its own capable of infecting one or more different plant species [Agrios, 1988; Goto, 1992].

This thesis is in fact the result of two independent projects which had in common the use of genetic modification to engineer bacterial disease resistance into plants. The first project entitled "Protection of *Solanaceae* against phytopathogenic microorganisms by the introduction of synthetic hordothionin genes", financed by the Dutch Programme Committee Agricultural Biotechnology (PcLB), was aimed at studying the potential of thionin encoding sequences for engineering bacterial disease resistance into solanaceous crops. The second project entitled "Protection of willow against the watermark disease by the expression of antibacterial proteins", financed by the arboriculture companies Boomkwekerij Udenhout B.V. and Ton van den Oever B.V., and the Dutch Programmatic Business orientated Technology Stimulation Fund (PBTS), was aimed at engineering resistance into willow against *Erwinia salicis*, the causal agent of the watermark disease. During the second project the potential of cecropin B encoding sequences for engineering resistance was investigated. In both projects tobacco and/or tomato, crops for which reliable transformation procedures had been developed in the past, were chosen as model plants. Construction and expression of the genes, processing of precursor proteins, sorting, biological activity and resistance of transgenic plants to phytopathogenic bacteria were investigated.

At the beginning of the first research project (beginning 1988), no engineered resistance against plant pathogenic bacteria had been reported. Our choice to study the feasibility of hordothionin genes was based on the reported toxicity of the thionin from wheat endosperm (purothionin) for plant pathogenic bacteria *in vitro* [Fernandez de Caleya *et al.*, 1972] and the presumption that expression of plant-derived genes in another plant species would not cause serious problems. The best characterized thionins at the molecular level were the hordothionins from barley endosperm, for which three cDNA sequences had been published before which indicated that these proteins were initially made as much larger precursors [Ponz *et al.*, 1986; Hernández-Lucas *et al.*, 1986]. The availability of these data and the high overall homology between purothionins and hordothionins led to our choice of the latter, although toxicity of the hordothionin for plant pathogenic bacteria had not been tested and the mode of action of thionins was unclear.

At the beginning of the second research project (middle of 1990), it was established that the hordothionins exhibited *in vitro* toxicity for few bacteria only. *Erwinia* species causing serious damage on solanaceous crops and a number of other plants (for example on willow) were not affected by these proteins *in vitro*, and this led us to investigate the applicability of cecropin B from the giant silkmoth, which had been reported to exhibit toxicity against numerous Gram-positive and Gram-negative bacteria [reviewed by Boman and Hultmark, 1987].

Chapter 1 reviews the literature available on thionins and cecropins. The different thionin types, their structure, reported toxicities and other activities are described. Possible mechanisms of action and biological roles are discussed. In the second part of chapter 1, the structure, toxicity and mechanism of action of cecropins are discussed. Special emphasis is put on one of these insect proteins, namely cecropin B which was under investigation in the

second research project.

Chapter 2 describes the isolation of purothionins and hordothionins from wheat and barley flour respectively, to establish their toxicity for plant pathogenic bacteria. Data are presented on the toxicity of both for bacteria causing serious damage on important crops.

Chapter 3 describes the design and construction of synthetic genes encoding different precursor hordothionins. Since no hordothionin cDNA clones were available to us and the PCR-technique was still in its infancy, we decided to chemically synthesize hordothionin encoding sequences, making use of the many advantages of synthetic genes. Gene constructs coding for α- and ß-hordothionin precursor proteins were made and optimized for expression in solanaceous crops by adapting the codon usage and translation initiation region. A cost-saving and reliable procedure for the chemical synthesis of genes is presented.

Chapter 4 describes the introduction and expression of these genes in tobacco. Data are presented on the effects of pre- and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein accumulation and sorting. In addition, data are presented which establish *in vitro* toxicity of these hordothionins from transgenic tobacco plants for one of the bacteria previously found to be sensitive. As a supplement, data on the growth of *Pseudomonas syringae* pv. *tabaci* in leaves of transgenic tobacco are presented.

Chapter 5 describes the introduction and expression of a full-length α-hordothionin gene construct in tomato. This gene construct was chosen because it resulted in high hordothionin accumulation when expressed in tobacco. Data on mature protein accumulation in leaves and fruits of transgenic plants are presented. In addition, the results of experiments to determine resistance of these transgenic tomato plants to plant pathogenic bacteria are given.

Chapter 6 describes the expression of three genes, coding for different cecropin B precursors, in tobacco. Data are presented on the stability of the antibacterial cecropin B peptide in different extracts of tobacco. In addition, the results of experiments to determine resistance of these transgenic tobacco plants to plant pathogenic bacteria are presented. The feasibility of using cecropin B encoding sequences for engineering resistance is discussed.

Finally, in chapter 7 a general discussion on the topic described here and a summary of the results are presented.

References

Agrios GN: Plant pathology. Third edition. Academic Press, Inc., San Diego (1988).

Boman HG, Hultmark D: Cell-free immunity in insects. Ann Rev Microbiol 41: 103-126 (1987).

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).

Goto M: Fundamentals of bacterial plant pathology. Academic Press, Inc., San Diego (1992).

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).

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).

Sawyer RL: Potatoes for the developing world. International Potato Center, Lima, Peru (1984).

CHAPTER 1

Thionins and cecropins

1.1 Introduction

In this thesis, the feasibility of two antibacterial proteins, the hordothionins from barley and cecropin B from the giant silkmoth, for engineering bacterial disease resistance into crops, is investigated. In this chapter, biochemical and biological activities which might be of importance to put thionins and cecropins into practice, will be discussed.

In the first part of this chapter, the thionins from plants will be discussed (§ 1.2). These were discovered in 1940 while searching for the substance present in wheat flour causing inhibition of fermentation and death of yeasts. This substance turned out to be toxic not only for yeasts, but also for bacteria, fungi and cells of diverse origins. I will focus on the different thionin types characterized, their occurrence and the structural homology between them (§ 1.2.1), synthesis and processing in plants from precursor proteins (§ 1.2.2), structure (§ 1.2.3) and toxicity (§ 1.2.4). Special emphasis will be put on the divergent activities observed for thionins *in vitro*, to obtain more insight in possible mechanisms of action underlying these properties, which are discussed in § 1.2.5. Possibilities for a biological role, other than a direct involvement in defense through the inhibition of growth of pathogens, as has been suggested by several authors, will also be discussed (§ 1.2.6). More general reviews on thionins have been published elsewhere [Apel *et al.*, 1990; Bohlmann and Apel, 1991; García-Olmedo *et al.*, 1989, 1992].

In the second part of this chapter, the cecropins from animals will be discussed (§ 1.3). Cecropins were first identified in 1980 as the major proteins present in the hemolymph of immunized pupae of the giant silkmoth [Hultmark *et al.*, 1980]. These proteins appeared to be bactericidal. Their antibacterial activity towards a number of Gram-positive and Gram-negative bacteria, and the triggering of cecropin gene expression by injection with living non-pathogenic or heat-killed pathogenic bacteria, suggested that this class of proteins might be involved in the cell-free immune response of insects. I will focus on the different cecropins characterized, their occurrence and the structural homology between them (§ 1.3.1), synthesis in insects from precursor proteins (§ 1.3.2), molecular structure (§ 1.3.3), toxicity (§ 1.3.4), mode of action (§ 1.3.5) and finally, their role in nature (§ 1.3.6). Reviews on cell-free immunity in insects and antibacterial peptides involved have been published elsewhere [Boman, 1991; Boman and Hultmark, 1987].

1.2 Thionins

Almost one hundred years ago, Jago and Jago suggested that wheat flour contained a substance lethal to brewer's yeast [cited in Wada *et al.*, 1982]. Half a century later this led to the discovery of purothionins [Balls and Hale, 1940]. These were recovered from a petroleum-ether extract of wheat flour after hydrochloric-acid treatment and turned out to have antimicrobial properties [Balls *et al.*, 1942; Stuart and Harris, 1942]. In the Middle Ages, the European mistletoe *Viscum album* was already known in folk medicine to contain a blood pressure lowering substance that could lead to death [cited in Rosell and Samuelsson, 1966; Samuelsson, 1973; Vernon *et al.*, 1985]. It took several ages before this activity was ascribed to a group of small proteins, the viscotoxins [Winterfeld and Bijl, 1949]. Later, these turned out to be highly homologous with the thionins from wheat. Afterwards, homologous proteins have been found in other species belonging to the *Poaceae*, in several mistletoe species and in the Abyssinian cabbage. All were collectively named thionins after the greek word for sulphur, describing one of the intrinsic properties of these proteins, namely the presence of a high amount of sulphur-containing cysteine residues [García-Olmedo *et al.*, 1989].

1.2.1 Types of thionins

Thionins can be divided into at least four different types depending on the plant species and tissues in which they occur, the overall net-charge and the number of amino acids and disulphide bonds present in the mature protein [García-Olmedo *et al.*, 1989]. The type 1 thionins are abundantly present in the endosperm of most *Poaceae*. They are highly basic and consist of 45 amino acids, eight of which are cysteins, involved in four disulphide bonds. Type 1 thionins can be obtained as lipoprotein complexes by petroleum-ether extraction of flour. Type 2 thionins have been extracted from the leaves and nuts of the parasitic plant *Pyrularia pubera* [Vernon *et al.*, 1985], and from barley (*Hordeum vulgare*) leaves [Bohlmann and Apel, 1987; Bohlmann *et al.*, 1988]. They consist of 47 or 46 amino acids respectively, are slightly less basic than the type 1 thionins, and also have four disulphide bonds. Type 3 thionins have been extracted from leaves and stems of a number of mistletoe species (*Viscum album*, *Phoradendron* spp. and *Dendrophtora clavata*). They consist of 46 amino acids with three disulphide bonds and are as basic as the type 2 thionins [Mellstrand and Samuelsson, 1973; Samuelsson, 1973; Samuelsson and Pettersson, 1977]. Type 4 thionins have been extracted from seeds of the Abyssinian cabbage (*Crambe abyssinica*). They consist of 46 amino acids with three disulphide bonds, but are neutral in charge [Van Etten *et al.*, 1965].

All members of these classes appear to be highly homologous at the amino acid level (Figure 1). Especially the cysteine residues, and apart from the type 4 thionins the tyrosine residue at position 13 and to a lesser extent the positively charged lysine and arginine residues, are highly conserved among all members. Castagnaro *et al.* [1992] reported another type of thionins, probably generated by mutation of the mature protein domain of a type 1 thionin. However, this thionin and the thionins collectively designated as τ-thionins in the literature [Colilla *et al.*, 1990; Gu *et al.*, 1992; Mendez *et al.*, 1990], do not share the overall homology described above. The type 4 thionins (crambins), which in contrast to type 1, 2 and 3 thionins do not have positively charged amino acids and a tyrosine at position 13, have been included because three dimensional structure analysis indicated that these proteins have a similar conformation as the type 1, 2 and 3 thionins [Clore *et al.*, 1987; Hendrickson and Teeter, 1981; Teeter and Whitlow, 1988; Whitlow and Teeter, 1985].

Nowadays, type 1, 2, 3 and 4 thionins are accepted as a typical family or class of low molecular weight proteins sharing high sequence homology, and an algorithm has been devised to characterize proteins belonging to this class. The consensus pattern which is used by the Prosite program (IntelliGenetics, Inc., GENOFIT SA., Geneva, Switzerland) to identify putative members is: C-C-x5-R-x2-(F,Y)-x2-C [Bairoch, 1989]. It includes three of the cysteine residues involved in disulphide bonds, an arginine residue and the tyrosine residue at position 13 (phenylalanine in crambin), which are conserved among the thionins listed in Figure 1.

Type	Name	Species	Tissue	Sequence[1]	pI[2]
				1 10 20 30 40 47	
1	α-hordothionin	*Hordeum vulgare*	endosperm	KSCCRSTLGRNCYNLCRVRGAQKL--CAGVCRCKLTSSGKCPTGFPK	10.06
	ß-hordothionin	*Hordeum vulgare*	endosperm	.....--..NA.....GL...SS...	10.06
	α1-purothionin	*Triticum aestivum*	endosperm	.....T.....S.....--.ST.....GLS..K....	10.06
	α2-purothionin	*Triticum aestivum*	endosperm	.....A.....--.....IS.GLS..K....	10.06
	ß-purothionin	*Triticum aestivum*	endosperm	....K.....A.....--..N.....GLS..KD...	9.64
	α-avenothionin	*Avena sativa*	endosperm	.....D....D..D...S...P..--..TL....IS.GLS..KD...	8.73
	ß-avenothionin	*Avena sativa*	endosperm	....KD....D..D...A...P..--.STL....I..GLS..KD...	8.73
	secalethionin	*Secale cereale*	endosperm	....K.....D..D...G...E..--..EL....I..GLS..KD...	8.51
2	leaf thionin (DB4)	*Hordeum vulgare*	leaves	....KD..A.....T.HFA.GSRPV-...A....II.GP...SDY..	8.72
	leaf thionin (DG3)	*Hordeum vulgare*	leaves	....KN.T.....A..FA.GSRPV-..TA.G..II.GPT..RDY..	8.96
	leaf thionin (BTH6)	*Hordeum vulgare*	leaves	....KD..A.....T..FA.GSRPV-...A....II.GP...SDY..	8.96
	Pyrularia thionin	*Pyrularia pubera*	leaves, nuts	.....N.WA.....V..LP.TISREI..KK.D..II.GTT..SDY..	8.72
3	Viscotoxin A2	*Viscum album*	leaves, stems	....PN.T...I..T..FG.GSR-EV..SLSG..II.AST..SYPD.	8.56
	Viscotoxin A3	*Viscum album*	leaves, stems	....PN.T...I..A..LT..PR-PT..KLSG..II.GST..SYPD.	9.17
	Viscotoxin B	*Viscum album*	leaves, stems	....PN.T...I..T..LG.GSR-ER..SLSG..II.AST..SYPD.	8.84
	Denclatoxin	*Dendrophtora clavata*	leaves, stems	....PT.AA..Q..I..LP.TPR-PV..ALSG..II.GTG..P.YRH	9.18
	Ligatoxin	*Phoradendron liga*	leaves, stems	....P..TA..I..T..LT.TSR-PT..SLSG..II.GST.BS.WBH	8.86
	Phoratoxin	*Phoradendron tomentosum*	leaves, stems	....PT.TA..I..T..FG.GSR-PV..KLSG..II.GT..DS.WNH	9.2
4	Crambin A	*Crambe abyssinica*	seeds	TT..P.IVA.SNF.V..LP.TPE-AI..TYTG.IIIPGAT..GDYAN	5.94
	Crambin B	*Crambe abyssinica*	seeds	TT..P.IVA.SNF.V..LP.TSEA.-..TYTG.IIIPGAT..GDYAN	5.94

Disulphide bonds[4]

Consensus[3] (100%) KSCC..T..R..Y..C...G.....C.....CK..S...C.....

Figure 1. Alignment of amino acid sequences of the members of the four different types of thionins currently available from the EMBL and SWISS-PROT databases. The one-letter code for amino acids is used and identical amino acids are represented by dots (.). Notes: [1] Sequences were derived from: α- and ß-hordothionin [Hernández-Lucas *et al.*, 1986]; α1-, α2- and ß-purothionin [Jones and Mak, 1976; Mak and Jones, 1976; Ohtani *et al.*, 1975; Ohtani *et al.*, 1977]; α- and ß-avenothionin [Békés and Lásztity, 1981]; secalethionin [Békés *et al.*, 1982]; leaf thionin (DB4, DG3 and BTH6) [Bohlmann and Apel, 1987; Bohlmann *et al.*, 1988]; Pyrularia thionin [Vernon *et al.*, 1985]; viscotoxin A2, A3 and B (Olson and Samuelsson, 1972; Samuelsson, 1973; Samuelsson and Pettersson, 1971]; ligatoxin [Thunberg and Samuelsson, 1982]; denclatoxin [Samuelsson and Pettersson, 1977]; phoratoxin [Mellstrand and Samuelsson, 1974a]; crambin A and B [Teeter *et al.*, 1981; Vermeulen *et al.*, 1987]. [2] Isoelectric point as predicted from the amino acid sequence. [3] The consensus sequence has been derived from a comparison of type 1, 2 and 3 thionins. [4] Disulphide bonds proven for phoratoxin [Mellstrand and Samuelsson, 1974b] and purothionins [Hase *et al.*, 1978] are represented above and below the consensus in boldface.

1.2.2 Synthesis and processing of thionins

Sequence analyses of cDNA's of type 1 [Hernández-Lucas *et al.*, 1986; Ponz *et al.*, 1986; Rasmussen and Rasmussen, 1993], type 2 [Andresen *et al.*, 1992; Bohlmann and Apel, 1987; Gausing, 1987] and type 3 thionins [Schrader and Apel, 1991], indicated clearly that these proteins are synthesized as much larger precursors. All these precursor thionins consisted of three distinct domains: an amino-terminal (N-terminal) signalpeptide involved in transfer of the precursor into the lumen of the endoplasmic reticulum (ER), the mature thionin and a carboxy-terminal (C-terminal) acidic peptide of unknown function. The typical structure of such a thionin precursor protein, is depicted in Figure 2. Sequences of cDNA's (and genomic clones) of type 4 thionins have not (yet) been reported.

Figure 2. The typical structure of a thionin precursor protein, consisting of an amino-terminal signalpeptide, the mature thionin and a carboxy-terminal acidic peptide.

Analyses of genomic clones of type 1 [Rodriguez-Palenzuela *et al.*, 1988] and type 2 thionins from barley [Bohlmann *et al.*, 1988] indicated that the genes contained two introns, both interrupting the C-terminal acidic peptide coding sequence. A maximum of 2-4 copies of the type 1 hordothionin gene per haploid genome was determined by Southern analysis [Rodríguez-Palenzuela *et al.*, 1988]. Genetic analyses indicated that the genes for the type 1 purothionins were localized on chromosome 1 of wheat, and that the amount of petroleum-ether-extractable thionin was also affected by a gene(s) on the short arm of chromosome 5 of wheat [Fernandez de Caleya *et al.*, 1976]. On the contrary, at least 50-100 copies of the type 2 barley leaf thionin gene per haploid genome were determined by Southern analysis, and all were confined to chromosome 6 of barley [Bohlmann *et al.*, 1988].

The type 1 thionins from barley are synthesized in developing endosperm from approximately 8 to 30 days after anthesis with maximum messenger concentrations at 13 to 16 days after pollination. No messengers were detected in either etiolated or green coleoptiles [Ponz *et al.*, 1983; Rodríguez-Palenzuela *et al.*, 1988]. The promoter of this barley endosperm thionin gene contains besides a TATA box and CATC boxes, several boxes and enhancer-like sequences homologous to sequences present in promoters of other endosperm genes [Rodríguez-Palenzuela *et al.*, 1988]. One of the major messengers present in young seedlings whose concentration rapidly declined upon illumination appeared to code for the type 2 thionins present in barley leaves [Gausing, 1987; Bohlmann and Apel, 1987]. Leaf thionin-mRNA levels were highest in the hypocotyledon and expression of the gene was triggered by pathogens [Bohlmann *et al.*, 1988], chemical stress [Fisher *et al.*, 1989] and by jasmonic acid [Andresen *et al.*, 1992].

The type 1 thionin precursor from barley endosperm is synthesized on membranebound polysomes and is processed in at least two steps suggesting cotranslational removal of the signalpeptide and posttranslational processing of the acidic peptide [Ponz *et al.*, 1983]. In sucrose gradient centrifugation, the hordothionin was found to cosediment with an ER marker and the protein bodies, but could be extracted from the particulate fraction without disturbing the protein body integrity [Carbonero *et al.*, 1980; Ponz *et al.*, 1983]. Recently, immuno-gold-labelling of thin sections from barley endosperm indicated specific labelling in the

periphery of the protein bodies using a hordothionin antiserum. At 13 days after anthesis the hordothionin was localized only in dense spheroids around the protein bodies, whereas at later stages hordothionin could also be detected throughout the membrane-like structure surrounding the protein bodies [Carmona *et al.*, 1993]. The leaf thionins were localized in the cell wall and vacuole of barley leaves [Bohlmann *et al.*, 1988; Reimann-Philipp *et al.*, 1989a,b], and were not found in the intercellular spaces of barley leaves [Fisher *et al.*, 1989]. Besides the transient increase of leaf thionin-mRNA upon infection of barley with spores of powdery mildew, *Erysiphe graminis* f.sp. *hordei* [Bohlmann *et al.*, 1988], cultivar-related differences in distribution of these thionins in epidermal cells in compatible and incompatible interactions between barley and powdery mildew have been reported [Ebrahim-Nesbat *et al.*, 1989]. These studies indicated that the type 1 and 2 thionins from barley are not secreted from the cell. The subcellular localization of the other types of thionins has not been reported.

1.2.3 Structure of thionins

Primary structure determinations revealed that approximately 12 to 17% of the amino acids making up thionins are cysteine residues. In all thionins six of these cysteine residues are conserved (Figure 1) and have been shown to be involved in disulphide bond formation [Hase *et al.*, 1978; Mellstrand and Samuelsson, 1974b]. In addition, the distribution of hydrophobic and hydrophilic residues among the different members is also conserved.

The crystal structure of crambin, a type 4 thionin isolated from *C. abyssinica* containing three disulphide bonds but no overall positive charge, was the first to be resolved (at 1.2 Å resolution) and was determined directly from the anomalous scattering of sulphur [Hendrickson and Teeter, 1981]. A similar secondary structure was predicted from circular dichroism (CD) data of crambin in phospholipid vesicles, indicating that the X-ray structure of thionins provided a detailed view of the molecular structure of the thionin as found in a membrane bilayer [Wallace *et al.*, 1984], which is the site at which these proteins are supposed to expose their toxicity (see § 1.2.5). CD and ^{1}H NMR studies showed that α1-purothionin, viscotoxin A3 and crambin had similar secondary structures [Clore et al., 1987; Teeter and Whitlow, 1988; Whitlow and Teeter, 1985]. ^{1}H NMR studies also indicated that α1-, α2- and ß-purothionin and α- and ß-hordothionin were structurally similar and were flexible [Lecomte *et al.*, 1982]. The tertiary structure in solution [Clore *et al.*, 1986], and crystal structure of α1-purothionin [Teeter *et al.*, 1990], confirmed previous predictions. These studies and others [Lecomte *et al.*, 1987; Teeter *et al.*, 1981; Vermeulen *et al.*, 1987] confirmed that type 1, 3 and 4 thionins are structurally related proteins.

The molecular conformation of α1-purothionin can be represented by the Greek capital letter gamma (Γ) and is depicted in Figure 3 [modified from Teeter *et al.*, 1990 and Whitlow and Teeter, 1985]. The vertical stem is an anti-parallel pair of α-helices and the horizontal arm consists of a strand and short anti-parallel ß-sheet (Figure 3a). Numerous salt bridges, hydrogen bonds and other contacts stabilize the structure and the four disulphide bridges further fix the internal structure of the sub-domains. The surface of α1-purothionin has an amphipathic character (Figure 3b). All charged groups and several neutral polar side chains are at the inner bend between the helical stem and the ß-arm and are clearly separated from the hydrophobic left side of the helical stem.

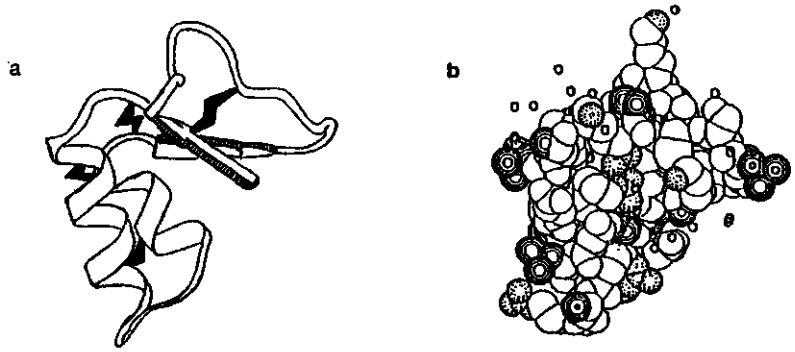

Figure 3. Three-dimensional structure of α1-purothionin [modified from Teeter *et al.*, 1990 and Whitlow and Teeter, 1985]. a, Schematic drawing of the backbone. Arrows depict ß-strands and disulphide bonds are indicated by solid lightning flashes. b, Surface diagram of α1-purothionin. Charged groups are shaded.

1.2.4 Antimicrobial activities of thionins in vitro

The purothionins were discovered in 1940 while searching for the substance causing a reduction of the baking quality of wheat flour. As soon as the substance was obtained in a crystalline form, it was tested for toxicity against a number of microorganisms and at relatively low concentrations, it appeared to be toxic for yeasts, bacteria and fungi *in vitro* [Stuart and Harris, 1942]. Afterwards, other thionins were also tested for antimicrobial activity. In Table 1, the reported *in vitro* toxicities of thionins for microorganisms (yeasts, fungi and bacteria), as deduced from growth inhibition experiments in solid and liquid media, are listed. Although the results are difficult to compare because of the different ways of testing, different purities of the proteins and dependance on the composition of the media used for testing (see § 1.2.5), doses indicate a rough estimate of the concentration range at which thionins are toxic.

In general, thionins appear to be toxic for a small spectrum of bacteria with no special preference for Gram-positive or Gram-negative bacteria. No *in vitro* growth inhibition experiments with type 3 and 4 thionins have yet been reported. In contrast to the small toxic spectrum against bacteria, thionins appear to have a broad toxic spectrum against fungi in culture [Cammue *et al.*, 1992]. Besides their *in vitro* toxicity for microorganisms, a large number of other *in vitro* and *in vivo* activities have been ascribed to thionins. These will be discussed in the next paragraph in relation to mechanisms of action.

Table 1. Toxicity of thionins for microorganisms

Type	Name	Dose	Organism and reference
1	purothionin	1-50 μg/ml	*Debaryomyces nadiformis*, *Eberthella typhi*, *Endomycopsis albicans*, *Pneumococcus Type I*, *Pneumococcus Type III*, *Saccharomyces cerevisiae* (bakery, brewery, winery), *Sarcina lutea*, *Staphylococcus aureus*, *Streptococcus viridans* [Stuart and Harris, 1942]
	purothionin	20 μg/ml	*Lactobacillus casei* [Wooley and Krampitz, 1942]
	purothionin	60-100 μg/ml	*Aspergillus flavus* [Baute and Richir, 1966]
	purothionin	1-110 μg/ml	*Corynebacterium flaccumfaciens*, *Corynebacterium poinsettiae*, *Corynebacterium sepedonicum*, *Pseudomonas solanacearum*, *Xanthomonas campestris*, *Xanthomonas phaseoli* [Fernandez de Caleya *et al.*, 1972]
	purothionin	4-16 μg/ml	*Saccharomyces* spp. (wild strains), *Saccharomyces cerevisiae*, *Saccharomyces uvarum* [Hernández-Lucas *et al.*, 1974]
	ß-purothionin	0.5-15 μg/ml	*Alternaria brassicola*, *Ascochyta pisi*, *Botrytis cinerea*, *Colletotrichum lindemuthianum*, *Fusarium culmorum*, *F. oxysporum* f.sp. *pisi*, *Nectria haematococca*, *Phoma betae*, *Pyrenophora tritici-repentis*, *Verticillium dahliae*, *Venturia inaequalis*, *Bacillus megaterium*, *Sarcina lutea* [Cammue *et al.*, 1992]
	hordothionin	50 μg/well	*Drechslera teres*, *Thielaviopsis paradoxa* [Bohlmann *et al.*, 1988]
2	barley leaf thionin	50 μg/well	*Drechslera teres*, *Thielaviopsis paradoxa* [Bohlmann *et al.*, 1988]
	Pyrularia thionin	62-125 μg/ml	*Micrococcus luteus* [Evett *et al.*, 1986]

1.2.5 Other features of thionins and mechanisms of action

The many reported activities of thionins displayed *in vivo* and *in vitro* will be discussed in the next paragraphs (§ 1.2.5.1 to § 1.2.5.5). A large number of these activities, and the antimicrobial activity of thionins displayed *in vitro* (§ 1.2.4), might be the result of interference with a mechanism common to all. This target of thionins will be proposed and discussed in view of the observed activities, in paragraph § 1.2.5.4. The results described in these paragraphs will be summarized in Table 2. Wherever possible, the dose at which the activity was found is indicated. In addition, the mechanism of action which might underlie the observed phenomena, is also indicated.

1.2.5.1 Electrostatic interaction with phospholipids

Acetylation of the positively charged groups of purothionin reduced the toxicity proportional to the number of modified groups [Wada *et al.*, 1982]. This might be explained by a reduction of the electrostatic interaction of the modified purothionin with the negatively charged polar tail groups of the lipids in the membrane. In fact, already in 1942, two years after the discovery of purothionins, it was reported that the antimicrobial effect of purothionin on *Lactobacillus casei* could be inhibited by simultaneously applying phospholipids [Wooley and Krampitz, 1942]. These phospholipids, mainly lipositol, lecithin and phosphatidylserine probably bind to the positively charged purothionin, resulting in the formation of a lipoprotein. The formation of such a complex prevents an electrostatic interaction with the negatively charged phospholipids present in the membrane of *L. casei*, which is a crucial step in the exposure of toxicity. The involvement of such an electrostatic interaction is further strengthened by the fact that all thionins exhibiting antimicrobial activity, have a net positive charge (Figure 1).

The involvement of purothionins in lipoprotein complexes in cereal endosperm, was later named lipopurothionin [Redman and Fisher, 1968]. The lipoprotein complexes, which could be isolated by petroleum-ether extraction of cereal flours, appeared to contain mainly polar lipids, among which phosphatidylethanolamine, phosphatidylcholine and digalactosyldiglyceride were the most prominent [Békés, 1975, 1981; Békés and Smied, 1981]. These phospholipids probably bind to the positively charged lysine and arginine residues of the thionin, thereby accounting for the solubility in petroleum-ether.

1.2.5.2 Interaction with specific domain in lipid membrane

Purothionin appeared to be toxic only in the S-phase (DNA-synthesis phase) of the cell cycle of animal cells and did not affect contact-inhibited cells [Nakanishi *et al.*, 1979]. Purothionin bound to the cell wall of sensitive yeast strains, but not to the cell wall of an insensitive strain or to the membranes of intracellular granules [Okada and Yoshizumi, 1973]. On the contrary, it bound to the protoplast membranes of both sensitive and insensitive strains. In the presence of Ca^{2+}, binding of purothionin to the membrane was not affected, although toxicity was completely inhibited in the presence of Ca^{2+}. These results suggest that binding to the membrane does not *per se* result in toxicity and that these are two separate steps in the mechanism of action. Purothionin and Ca^{2+} probably compete for the same domain on the membrane surface, which might not be common to all biological membranes. Experiments in which the sole tyrosine residue of purothionin (position 13; Figure 1) was modified confirmed that binding to the membrane and exposure of toxicity are two different steps. Modification of the tyrosine residue of purothionin drastically reduced or eliminated toxicity

[Wada *et al.*, 1982], although this modification did not affect the overall charge of the protein and hence the electrostatic interaction with the phospholipids in the membrane. The importance of tyrosine in toxicity of thionins was also shown for Pyrularia thionin. Unmodified Pyrularia thionin activates endogenous phospholipase A2 in cultured mouse cells and is hemolytic in case of erythrocytes (see also § 1.2.5.4). Pyrularia thionin furthermore depolarized membranes of various origins. All these activities were lost upon iodination of this thionin which affected the state of the tyrosine residue [Evans *et al.*, 1989]. These results implicated that besides a positive charge, a tyrosine residue is indispensable for toxicity. Interestingly, it is conserved among all thionins with the exception of the type 4 thionin (Figure 1), which as stated before shows no toxicity. This tyrosine residue might thus be involved in receptor binding. In fact, the interaction of thionin with a specific receptor in the membrane was confirmed and extended by investigations into the interaction of Pyrularia thionin and human and other mammalian erythrocytes.

Pyrularia thionin was capable of lysing human erythrocytes at 10 μg/ml [Evett *et al.*, 1986]. Subsequent experiments indicated that hemolysis followed Michaelis-Menten kinetics and was inhibited by Ca^{2+} concentrations above 1 mM [Osorio e Castro *et al.*, 1989]. These results indicate the involvement of a specific receptor on the membrane. Hemolytic activity of Pyrularia thionin was also shown for erythrocytes derived from various other mammals. For these, hemolysis had different K_m values [Osorio e Castro and Vernon, 1989; Osorio e Castro *et al.*, 1990]. In addition, iodinated Pyrularia thionin was not hemolytic, but competed for the same receptor on the membrane with native Pyrularia thionin and snake venom cardiotoxin [Osorio e Castro and Vernon, 1989; Vernon and Rogers, 1992a,b]. The inhibition of the hemolytic activity of Pyrularia thionin by Ca^{2+} probably is the result of competitive binding to the same receptor. Furthermore, removal of Ca^{2+} from the medium prior to adding Pyrularia thionin to erythrocytes, increased hemolytic activity. Calculation of the number of Pyrularia thionin binding sites on the membrane of a single cell varied from 10^4 to 10^6 sites per cell, depending on the cell under investigation, and was proportional to the sensitivity of the cell towards thionin treatment [Osorio e Castro *et al.*, 1989; Vernon and Rogers, 1992b]. Searching for a proteinaceous receptor was however negative, and in view of the large number of receptor sites present on the membrane of a single cell, the receptor most likely is a specific phospholipid domain. The composition of this domain might be reflected by the phospholipids contained in the lipopurothionin complex (see § 1.2.5.1).

1.2.5.3 Pore-formation

Purothionin appeared to be toxic for yeasts at relatively low concentrations (4 μg/ml). Even at tenfold lower concentrations (0.4 μg/ml), a clear effect was established. Sugar uptake was inhibited and K^+, PO_4^{2-}, amino acids and nucleotides leaked from the cells [Okada and Yoshizumi, 1973]. These effects suggested the formation of pores, or a change in fluidity of the membrane. This was confirmed by experiments in which the antibiotic hygromycin B, which on its own is not capable of crossing membranes, was added to baby hamster kidney (BHK) cells together with sublethal concentrations of purothionin. At concentrations where the thionin alone did not affect translation in BHK cells, addition of small amounts of hygromycin B strongly inhibited translation, suggesting its uptake into the cell through the membrane [Carrasco *et al.*, 1981]. The capability of forming pores in biological membranes is furthermore supported by the crystal structure of purothionin (Figure 3), in which the amphipathic α-helices have hydrophobic groups directed towards one side of the molecule and hydrophilic groups towards the other side. The hydrophobic groups could interact with polar tail groups of lipids and the hydrophilic groups could extend towards the aqueous phase. Upon aggregation of more of these molecules, this could result in the formation of

lipid-thionin pores and disruption of the membrane [Teeter *et al.*, 1990]. However, besides amphipathy of the α-helices, a minimum of 20 residues is needed for membrane-spanning [Lear *et al.*, 1988]. The two anti-parallel amphipathic α-helices in purothionin supposed to be involved in pore-formation hence cannot form a membrane-spanning α-helix because of their small size, suggesting another mechanism of action resulting in disruption of the membrane.

1.2.5.4 Possible target in the membrane

In the presence of Ca^{2+}, Pyrularia thionin caused the depolarization of the plasma membrane of cultured mouse cells and of the sciatic nerve-sartorius muscle from a frog, *Rana pipiens* [Evans *et al.*, 1989]. Pyrularia thionin appeared to mediate an influx of Ca^{2+} into mouse cells, suggesting the activation of a calcium channel. Depolarization of these membranes by Pyrularia thionin could be inhibited by Ni^{2+}, a calcium channel blocker, and by the channel blocker verapamil. An effect on ion channels was confirmed by the release of prolactin and growth hormone from Pyrularia thionin-treated rat cells which depended on dopamine, which closes calcium channels, and D-600, an organic calcium channels blocker [Judd *et al.*, 1992].

Moreover, the type 3 thionins, viscotoxin and phoratoxin, appeared to cause a transient decrease in blood pressure and contractile force, whereas the heart rate decreased irreversibly upon intravenous injection of cats [Rosell and Samuellson, 1966]. This indicates that these substances exert a toxic effect directly upon the heart muscle. When administered at high concentrations, injection resulted in vasoconstriction of vessels in skin and skeletal muscle. These effects are most likely the result of the interference of these type 3 thionins with calcium channels, which are indispensable for muscle contraction, propagation of action potentials, maintenance of electrical activity and neurotransmitter regulation. The effects of these thionins on muscles, membranes and cardiac contractions resemble those of other highly basic peptides, which are also tightly reticulated by disulphide bonds. Calciseptine, a 60-amino acid peptide isolated from black mamba (*Dendroaspis polylepis polylepis*) venom abolishes contractions in cardiac and smooth muscle cells by blocking L-type calcium channels [De Weille *et al.*, 1991]. Some of the conotoxins from fish-hunting cone snails abolish muscle contraction by interacting with muscle sodium channels, whereas other conotoxins have affinity for potassium or calcium channels [reviewed in Olivera *et al.*, 1985; Woodward *et al.*, 1990]. Scorpion toxins depolarize membranes upon binding to them, specifically by blocking either calcium, sodium or calcium-activated potassium channels [Auguste *et al.*, 1992; Fontecilla-Camps *et al.*, 1981]. Snake venom toxins from cobra also depolarize membranes and relax muscles, and have been shown to compete with Pyrularia thionin for the same receptor on the erythrocyte membrane [Osorio e Castro and Vernon, 1989]. The resemblance in activity of thionins and these toxins and the competition of Ca^{2+} and Pyrularia thionin for the same receptor, make it very likely that thionins interact with calcium channels.

Such an interaction could explain many of the activities displayed by thionins *in vitro* (see Table 2). The efflux of K^+ and Mg^{2+} from thionin-treated yeast cells [Okada and Yoshizumi, 1973] and from adrenal bovine medullary cells [Kashimoto *et al.*, 1979], which by the authors was suggested to be the result of pore-formation by thionins, could have been caused by a cellular response to an increased Ca^{2+} influx, resulting from the activation of a calcium channel. Leakage of these ions and various other small molecules (such as nucleotides and amino acids) does not have to be the result of pore-formation, since ion-channels have been shown to be permeable for molecules other than the ions they transport [reviewed in Ranjeva *et al.*, 1993]. Hemolysis of human erythrocytes by Pyrularia thionin was accompanied by the release of free fatty acids, most likely due to the activation of endogenous phospholipases

[Angerhofer *et al.*, 1990; Evans *et al.*, 1989; Judd *et al.*, 1992]. An increased cellular Ca^{2+} level, caused by activation of a calcium channel, might result in the onset of the calcium signal transduction cascade and hence could activate endogenous phospholipases. This then could result in the release of free fatty acids, prolactin, growth hormone, arachidonate and arachidonic acid and inhibition of translation as the result of a change in fluidity of the membrane. A lot of these processes are actually activated in some way through Ca^{2+} [reviewed in Johannes *et al.*, 1991; Ranjeva *et al.*, 1993; Trewavas and Gilroy, 1991].

Because of the competition between Ca^{2+} and thionin for the same membrane receptor, the molecular structure of thionins (§ 1.2.3), leakage of ions and other small molecules from thionin-treated cells and resemblance to the other known proteinaceous toxins mentioned above, thionins most likely interact with the channel mouth near the channel pore.

1.2.5.5 Interaction with proteins and nucleic acid

Purothionin appeared to exhibit thioredoxin activity *in vitro*, by the conversion of S-S and S-H groups [Wada and Buchanan, 1981; Johnson *et al.*, 1987]. The inhibition of papainase and α-amylase [Balls *et al.*, 1942; Jones and Meredith, 1982], the reduction of purothionin by wheat seed thioredoxin and the inhibition of ribonucleotide reductase [Johnson *et al.*, 1987], most likely are also the result of the conversion of S-S groups of the enzyme into S-H groups (or *vice versa*) by thionin.

Viscotoxin appeared to prevent degradation of DNA by heating, as the result of binding to the DNA [Woynarowski and Konopa, 1980]. The binding was random and resulted from an electrostatic interaction between the highly positively charged viscotoxin and the negatively charged DNA. The inhibition of protein synthesis in an eukaryotic cell-free, wheat-germ translation system by purothionin, could be the result of direct binding of purothionin and RNA [García-Olmedo *et al.*, 1983], or inactivation of enzymes due to reduction by purothionin. The amount of purothionin present in diverse wheat-germs appeared to be directly correlated with the translation efficiency of these extracts.

Table 2. Listing of the various activities displayed by the different thionins and the possible mechanisms involved

Type	Name	Mechanism[1]	Dose	Activity and reference
1	purothionin	ion channel	1.6-6 mg/kg	toxic to mice, guinea pig and rabbit upon intravenous injection [Coulson *et al.*, 1941]
	purothionin	ion channel	0.8 μg/ml	contraction of isolated guinea pig uterus [Coulson *et al.*, 1941]
	purothionin	thioredoxin		inhibition papainase enzyme activity *in vitro* [Balls *et al.*, 1942]
	purothionin	ion channel	4 μg/ml	inhibition respiration and fermentation of intact yeast cells and protoplasts *in vitro* leading to death [Okada and Yoshizumi, 1973]
	purothionin	ion channel	0.4 μg/ml	inhibition sugar incorporation; leakage of K^+, PO_4^{2-}, protein and nucleotides from yeast cells *in vitro* [Okada and Yoshizumi, 1973]
	purothionin	ion channel	4 μg/ml	induction leakage of cytoplasmic proteins and Mg^{2+} ions from bovine adrenal medullary cells *in vitro* [Kashimoto *et al.*, 1979]
	purothionin	ion channel	19-46 μg/g	toxic to *Manduca sexta* larvae upon injection into the hemocoel [Kramer *et al.*, 1979]
	purothionin	ion channel	2-50 μg	depolarization muscle-fiber membrane of isolated nerve-flight muscle from *Manduca sexta* moth [Kramer *et al.*, 1979]
	purothionin	ion channel	6-10 μg/ml	cytotoxic for A-31 and hamster BHK cells *in vitro* [Nakanishi *et al.*, 1979]
	purothionin	ion channel	0.5-20 μg/ml	inhibition translation in hamster BHK-21 and monkey CV1 cells *in vitro* and leakage of $^{86+}$Rb from cells [Carrasco *et al.*, 1981]
	purothionin	thioredoxin		exhibition thioredoxin activity *in vitro* [Wada and Buchanan, 1981]
	purothionin	thioredoxin	50 μg/ml	inhibition α-amylase enzyme activity *in vitro* [Jones and Meredith, 1982]
	purothionin	RNA binding	25-35 μg/ml	inhibition cell-free translation in wheat germ and rabbit reticulocyte lysate [García-Olmedo *et al.*, 1983]
	purothionin	ion channel	20 μg/ml	cytotoxic for mosquito and spruce budworm cells *in vitro* [Jones *et al.*, 1985]
	purothionin	thioredoxin		reduction by wheat seed thioredoxin and inhibition of ribonucleotide reductase *in vitro* [Johnson *et al.*, 1987]
	purothionin	ion channel	100 μg/ml	release arachidonic acid from cultured mouse fibroblast cells [Angerhofer *et al.*, 1990]
	purothionin	ion channel	50 μg/ml	cytotoxic to human umbilical vein endothelial and skin-muscle fibroblasts *in vitro* [Cammue *et al.*, 1992]
	hordothionin	ion channel	17-45 μg/g	toxic to *Manduca sexta* larvae upon injection into the hemocoel [Kramer *et al.*, 1979]
	hordothionin	ion channel	0.5 μg/ml	inhibition translation in hamster BHK-21 cells *in vitro* [Carrasco *et al.*, 1981]
	secalethionin	ion channel	42 μg/g	toxic to *Manduca sexta* larvae upon injection into the hemocoel [Kramer *et al.*, 1979]
2	leaf thionin	ion channel	25 μg/ml	toxic to cultured tobacco protoplasts [Reimann-Philipp *et al.*, 1989b].
	Pyrularia thionin	ion channel	7-40 μg/ml	cytotoxic to murine B16 melanoma and human HeLa cells *in vitro* [Evans *et al.*, 1989; Vernon *et al.*, 1985]
	Pyrularia thionin	ion channel	1.5 mg/kg	toxic to mice upon intraperitoneal injection [Evett *et al.*, 1986]
	Pyrularia thionin	ion channel	10 μg/ml	hemolysis human red blood cells *in vitro* [Evans *et al.*, 1989; Evett *et al.*, 1986; Osorio e Castro *et al.*, 1989; Vernon and Rogers, 1992a,b]
	Pyrularia thionin	ion channel	10-85 μg/ml	depolarization of plasma membrane of mouse P388 cells and frog sartorius muscle *in vitro* [Evans *et al.*, 1989]
	Pyrularia thionin	ion channel	100 μg/ml	activation phospholipases in cultured mouse fibroblast cells and human erythrocytes *in vitro* [Angerhofer *et al.*, 1990; Osorio e Castro *et al.*, 1989b]
	Pyrularia thionin	ion channel	100 μg/ml	release free fatty acids from cultured mouse fibroblast cells and human erythrocytes *in vitro* [Angerhofer *et al.*, 1990; Osorio e Castro *et al.*, 1989]

Table 2. continued

Type	Name	Mechanism[1]	Dose	Activity and reference
2	Pyrularia thionin	ion channel	0.1-10 μg/ml	release of prolactin and growth hormone from rat pituitary cells *in vitro* [Judd *et al.*, 1992]
	Pyrularia thionin	ion channel	0.1-10 μg/ml	liberation of arachidonate from rat pituitary cells *in vitro* [Judd *et al.*, 1992]
	Pyrularia thionin	ion channel	100 μg/ml	activation phospholipases in cultured mouse fibroblast and human erythrocytes [Angerhofer *et al.*, 1990; Osorio e Castro *et al.*, 1989]
3	viscotoxin	ion channel	17-35 μg/kg	bradycardia, negative inotropic effect on heart and vasoconstriction of vessels in skin and skeletal muscle of cats upon injection [Rosell and Samuelsson, 1966]
	viscotoxin	DNA binding		non-random binding to DNA [Woynarowski and Konopa, 1980]
	viscotoxin	ion channel	0.2-1.7 μg/ml	cytotoxic to human KB and HeLa cells *in vitro* [Konopa *et al.*, 1980]
	viscotoxin	ion channel	5 μg/ml	inhibition translation in hamster BHK-21 cells *in vitro* [Carrasco *et al.*, 1981]
	phoratoxin	ion channel	400 μg/kg	bradycardia, negative inotropic effect on heart and vasoconstriction of vessels in skin and skeletal muscle of cats upon injection [Rosell and Samuelsson, 1966]
	phoratoxin	ion channel	570 μg/kg	toxic to mice upon intraperitoneal injection [Mellstrand and Samuelsson, 1973]
	phoratoxin	ion channel	300 μg/ml	release arachidonic acid from cultured mouse fibroblast cells [Angerhofer *et al.*, 1990]

[1] Proposed mechanism of action, see text

1.2.6 Biological role of thionins

The reported *in vitro* antimicrobial toxicity of the type 1 thionins from wheat for a number of plant pathogenic bacteria [Fernandez de Caleya *et al.*, 1972] led the authors to suggest a possible role in protection of the plant against microbial attack [García-Olmedo *et al.*, 1989]. The reported *in vitro* toxicity of the barley leaf thionins for two plant pathogenic fungi [Bohlmann *et al.*, 1988], and the observed differences in distribution of these thionins in epidermal cells in compatible and incompatible interactions between barley and powdery mildew [Ebrahim-Nesbat *et al.*, 1989], also suggested that the type 2 barley leaf thionins were directly involved in defense against microbial (fungal) attack. However, considerable concentrations of the respective thionins were needed for a bactericidal or fungicidal effect (see Table 1), and hence a role in defense of the plant by inhibiting growth of intruding pathogens is not likely. In addition, none of the characterized fungal resistance genes from barley have been mapped in the neighborhood of the leaf thionin genes on chromosome 6 [Ebrahim-Nesbat *et al.*, 1989]. Leaf thionins might however have an indirect role in defense, for example by altering the cell wall upon penetration of the epidermis by fungal hyphen, or by triggering the signal transduction pathway.

Type 1 thionin gene expression and the subcellular localization of these proteins in endosperm (see § 1.2.2), suggest a role in seed-related activities. Since thionins can inhibit or activate enzymes, act as secondary thiol messengers, activate calcium channels and phospholipases (see Table 2), these proteins might have a role during seed maturation, dormancy or germination. A role in packaging storage proteins into protein bodies or mobilization during germination has been suggested by Carmona *et al.* [1993] and was based on the localization of thionins around the protein bodies. Especially a role in the latter, namely mobilization of endosperm, seems plausible. Endosperm mobilization in germinating cereal grains involves many complicated processes, including the proliferation of an intracellular membrane system [reviewed in Fincher, 1989]. Thionins might regulate some of these processes either directly, for example by inhibition or activation of enzymes, by acting as secondary thiol messengers (§ 1.2.5.5) or indirectly through Ca^{2+} (§ 1.2.5.4). With respect to the latter, thionins might activate calcium channels and hence regulate phospholipases or protein kinases, or other components of the signal transduction system. No expression and localization studies have been reported for the Pyrularia thionin and the type 3 and 4 thionins, besides their recovery from leaves, stems and seeds. Their biological role thus remains to be elucidated.

1.2.7 Concluding remarks and areas for further research

In conclusion, biologically active thionins are highly basic plant proteins, showing both sequence and structural homology. They are folded into rigid molecules that are stabilized by several disulphide bonds. They are toxic for a number of bacteria, fungi, yeasts and various naked cells *in vitro*. Toxicity requires an electrostatic interaction of the positively charged thionin with the negatively charged phospholipids making up the membrane and a specific interaction with a certain lipid domain, involving the conserved tyrosine residue. *In vitro* toxicity might be the result of an interference with calcium channels. Activation of calcium channels results in increased cellular Ca^{2+} levels, which subsequently results in the onset of the signal transduction cascade. Thionins are also capable of rearranging disulphide bonds and can bind nucleic acid. An involvement in plant defense by inhibiting pathogens has been suggested by several authors. A role other than a direct involvement in defense, is equally plausible in view of the many divergent activities observed. Definite proofs for the suggested

mechanisms of action and biological roles for thionins in the different species accumulating these proteins, awaits further testing. Their toxicity for plant pathogenic bacteria and fungi renders these proteins into potential tools for engineering resistance into plant species that lack these proteins. Expression studies in heterologous species might be a powerful tool for elucidating some of these uncertainties.

1.3 Cecropins

The presence of an inducible cell-free immunity in insects comprising antibacterial substances has been known for several decades [reviewed in Boman and Hultmark, 1987]. Injection of bacteria into the hemocoel of pupae of the giant silkmoth (*Hyalophora cecropia*) resulted in the recovery of the first pure antibacterial factors. Injection resulted in a rapid synthesis of RNA and specific proteins that gave rise to an increased antibacterial activity in the hemolymph [Hultmark *et al.*, 1980]. Among the newly synthesized proteins, two small, highly basic proteins were characterized in 1981 and named cecropins [Steiner *et al.*, 1981]. The antibacterial activity present in the hemolymph of these immunized pupae could largely be ascribed to these cecropins, although other proteins belonging to the attacins [Engström *et al.*, 1984a,b; Hultmark *et al.*, 1983; Kockum *et al.*, 1984] or lysozymes [Engström *et al.*, 1985] have also been identified to exhibit antimicrobial activity.

Cecropins exhibit toxicity to bacteria only, and do not affect eukaryotic cells [Steiner *et al.*, 1981]. After their discovery in the giant silkmoth, cecropin-like peptides (hereafter referred to as cecropins) were identified in numerous other insects [reviewed in Boman and Hultmark, 1987; Hultmark, 1993]. Cecropins play a key role in defense of the insect. Apart from insects, a homologous peptide was also identified in the intestine of pigs [Lee *et al.*, 1989]. Here, cecropins are believed to play a role in controlling the bacterial flora of the intestine.

1.3.1 Types of cecropins

Cecropins have been identified in numerous *Lepidoptera*, *Coleoptera* and *Diptera* and most likely they are also present in *Hemiptera*. The presence of cecropins among members of other genera is not (yet) known. Hereafter, the cecropins that have been purified and characterized by sequencing will be discussed. They will be identified by the names given to these peptides, as described in the original papers. The first letter of these will be in capital to avoid confusion when discussing cecropins in general.

The first cecropins purified and sequenced were Cecropin A and B from the lepidopteran *H. cecropia* [Steiner *et al.*, 1981]. In addition to these two, a third one, named Cecropin D was purified from *H. cecropia* and characterized [Hultmark *et al.*, 1982]. Homologous peptides, named Cecropin A and B, Antibacterial Peptide CM-IV, Lepidopteran A and B, were isolated from the common silkworm, *Bombyx mori* [Morishima *et al.*, 1990; Taniai *et al.*, 1992; Teshima *et al.*, 1986]. An additional two, Cecropin B and D, were isolated from the chinese oak silkworm moth, *Antheraea pernyi* [Qu *et al.*, 1982]. Cecropin A1, A2 and C were isolated from the dipteran fruitfly, *Drosophila melanogaster* [Kylsten *et al.*, 1990; Tryselius *et al.*, 1991]. Bactericidin B-2 through B-5 were isolated from the lepidopteran tobacco hornworm, *Manduca sexta* [Dickinson *et al.*, 1988]. Sarcotoxin IA through IC were isolated from the dipteran flesh fly, *Sarcophaga peregrina* [Matsumoto *et al.*, 1986; Okada and Natori, 1985b]. Cecropin 1 and 2 were isolated from the Mediterranean fruit fly, *Ceratitis capitata* [Rosetto *et al.*, 1993]. Cecropin P1 was isolated from pig (*Sus*

scrofa) intestine [Lee *et al.*, 1989].

All these cecropins consist of 31 to 39 amino acids, are basic, devoid of cysteine and share structural characteristics (Figure 4). Based on careful examination of their primary sequences, four classes might be distinguished. The first class would encompass the Cecropins A and B from *H. cecropia*, *B. mori* and *A. pernyi*, Lepidopteran A/B and Antibacterial Peptide CM-IV from *B. mori*; the second class would consist of Cecropin D from *H. cecropia* and *A. pernyi* and the Bactericidins from *M. sexta*; the third would encompass the Cecropins from *C. capitatis* and *D. melanogaster* and the Sarcotoxins from *S. peregrina*; the fourth would consist solely of Cecropin P1 from pig intestine (Figure 4). All cecropins appear to have an amidated carboxy-terminal amino acid residue, with the exception of Cecropin P1 from pig intestine.

The consensus pattern which is used by the Prosite program (IntelliGenetics, Inc., G-ENOFIT SA, Geneva, Switzerland) to identify putative members belonging to the cecropin family of proteins, W-x(0,2)-[KN]-x(2)-K-[KE]-[LI]-E-[RKN] [Bairoch, 1990], is designed based on the conserved amino terminus (see Figure 4).

1.3.2 Cecropin precursors and processing

Sequence analyses of cecropin cDNA's and genomic clones indicated that these proteins are synthesized as larger precursor proteins. Cecropin A and B from *H. cecropia* are synthesized as precursor proteins consisting of the mature cecropin part (36-38 amino acids), preceded by an additional 26 amino acids at the amino-terminus [Van Hofsten *et al.*, 1985; Lidholm *et al.*, 1987]. These amino acids resemble a signalpeptide, which is probably involved in transport of the precursor over membranes. With chemically synthesized Cecropin A and B precursor proteins, Boman *et al.* [1989a] showed that only the first 22 amino acids of these precursors were in fact a signalpeptide, and that processing occurred by a signal peptidase between Ala^{-5} and Ala^{-4} (with regard to the first amino acid of the mature cecropin). The resulting precursors were further processed in two steps by a specific dipeptidyl aminopeptidase (DPAP), which removed the dipeptides Ala-Pro (residues -4 and -3) and Glu-Pro (residues -2 and -1). The typical structure of the precursor of Cecropin B from *H. cecropia* is illustrated in Figure 5.

Name[1]	Species	Sequence[2]
Cecropin A	*Hyalophora cecropia*	KW--KLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-G
Cecropin B	*Hyalophora cecropia*	..---.V.....M.R...N..V.....I..L.E.--K.L-.
Cecropin B	*Antheraea pernyi*	..---.I.....R...N.....L.E.---KA-L
Cecropin B	*Bombyx mori*	R.---.L.....R.V...L.....I..I...KSL----
Lepidopteran A/B	*Bombyx mori*	R.---.I.....M.R.....V.....IE.L.S.KA.--GK
Antibacterial Peptide CM-IV	*Bombyx mori*	R.---.I.....V.....---.T-I
Cecropin D	*Hyalophora cecropia*	-.--NP..EL.....RV..AV.S.....T.A...AL..GK
Cecropin D	*Antheraea pernyi*	-.--NP..EL.RA..RV..A..S.....T.A...AL..--
Bactericidin B-2	*Manduca sexta*	-.--NP..EL.RA..RV..AV.S.A....T....AA..R-.
Bactericidin B-3	*Manduca sexta*	-.--NP..EL.RA..RV..A..S.....T....AA..R-.
Bactericidin B-4	*Manduca sexta*	-.--NP..EL.RA..RV..A..S.A....T....AA..R-.
Bactericidin B-5	*Manduca sexta*	-.--NP..EL.RA..RV..AV.S.-A...T....AA..RG.
Cecropin 1	*Ceratitis capitata*	G.LK.IG....R...HT..AT.QTIAVAQQAANVAAT.R-.
Cecropin 2	*Ceratitis capitata*	G.LK.IG....R...HT..AT.QTIGVAQQAANVAATL.-.
Cecropin A1/A2	*Drosophila melanogaster*	G.LK.IG....R...HT..AT.QGLGIAQQAANVAAT.R-.
Cecropin C	*Drosophila melanogaster*	G.LK..G.R..RIG.HT..AT.QGLGIAQQAANVAAT.R-.
Sarcotoxin IA	*Sarcophaga peregrina*	G.LK.IG....R...HT..AT.QGLGIAQQAANVAAT.R-.
Sarcotoxin IB	*Sarcophaga peregrina*	G.LK.IG....R...HT..AT.QVIGVAQQAANVAAT.R-.
Sarcotoxin IC	*Sarcophaga peregrina*	G.LR.IG....R...HT..AT.QVLGIAQQAANVAAT.R-.
Cecropin P1	*Sus scrofa*	S.----LS.TA.KLE.SAKKR.SE.I.-----I.I.GGP-R

Figure 4. Alignment of amino acid sequences of cecropins currently available from the EMBL or SWISS-PROT databases and grouped according to homology into four subclasses. The one-letter code for amino acids is used, hydrophilic residues are represented in boldface, identical amino acids by dots (. or .), and gaps by dashes (-). Notes: [1] the names of the cecropins, and [2] the amino acid sequences, are those described in the original papers discussed in § 1.3.1.

Figure 5. The typical structure of the precursor of Cecropin B from the giant silkmoth *H. cecropia*, consisting of an amino-terminal signalpeptide, followed by two dipeptides which are successively cleaved by a dipeptidyl aminopeptidase (DPAP), and the carboxy-terminal mature Cecropin B part.

All known cecropins have an amino-terminal signalpeptide in their precursor, although not all have two dipeptides which are processed by a DPAP. Cecropin B from *B. mori* probably has two dipeptides, Ala-Pro and Glu-Pro, which are subsequently removed by a DPAP [Kato *et al.*, 1993]. Bactericidin B5 from *M. sexta* [Dickinson *et al.*, 1988] and Cecropin D from *H. cecropia* [Gudmundsson *et al.*, 1991; Lidholm *et al.*, 1987] are believed to have one dipeptide, Ala-Pro. Sarcotoxin IA from *S. peregrina* [Matsumoto *et al.*, 1986] and the Cecropins A1, A2 and C from *D. melanogaster* [Kylsten *et al.*, 1990] probably do not have additional amino acids between the putative signalpeptidase cleavage site and the first amino acid residues of the mature cecropin. The amidated carboxy-terminal amino acid residue has to be derived by posttranslational conversion of a glycine residue into the next-to-last α-carboxamide residue, as apparent from the cloned sequences.

In *H. cecropia*, cecropins are most likely synthesized by fat body cells [Faye and Wyatt, 1980], and secreted into the hemolymph. In *M. sexta*, Bactericidin-RNA transcripts were also detected in the midgut, Malpighian tubules, the pericardial complex, epidermis and hemocytes [Dickinson *et al.*, 1988], whereas in *D. melanogaster* [Samakovlis *et al.*, 1990] and *B. mori* [Kato *et al.*, 1993], cecropin-mRNA's were detected only in fat body cells and hemocytes. These findings indicate that in addition to fat body cells, other tissues may also contribute to the synthesis of cecropins.

1.3.3 Cecropin molecular structure

Secondary structure analyses by circular dichroism (CD) experiments indicated that Cecropin A and B from *H. cecropia* exist largely as random coil structures when in dilute aqueous buffers, whereas in hydrophobic environments helical structures are adapted [Steiner, 1982]. Helical wheel representations of the amino acid sequences of these cecropins, indicate their potential to form two α-helices, one amphipathic α-helix and one more hydrophobic helix, both separated by a flexible hinge (see Figure 4 and 5). This structure was confirmed by ^{1}H NMR analysis. Two helical regions, extending from amino acid residues 5 to 21 and 24 to 37 were detected [Holak *et al.*, 1988]. All other cecropins can also form amphipathic α-helices, as apparent from helical wheel representations of their amino acid sequences. These structures facilitate interaction with biological membranes.

1.3.4 Antibacterial activity of cecropins in vitro

Cecropin synthesis is triggered by injection of living non-pathogenic or killed insect-

pathogenic bacteria into the hemolymph, or simply by wounding the insect. Because cecropin synthesis is transient and only specific organelles produce these peptides, a major role of these peptides in defense of the organism against microbial attack has been suggested. Most cecropins have been isolated and characterized as the major antibacterial constituents of the hemolymph of immunized insect pupae or larvae. Since cecropins can only be obtained in small amounts from hemolymphs, most of them were chemically synthesized to study their toxicity in more detail. Cecropins affected both Gram-positive and Gram-negative bacteria, however they showed a strong preference in toxicity for bacteria that are non-pathogenic to the organism from which these cecropins were isolated. The lethal concentrations ranged between 1 and 40 μg/ml (0.2-10 μM) for most of the sensitive bacteria [Andreu *et al.*, 1983; Boman *et al.*, 1989a; Fink *et al.*, 1989; Lee *et al.*, 1989; Qu *et al.*, 1982; Van Hofsten *et al.*, 1985]. Insect-pathogenic bacteria generally appeared to be less susceptible, suggesting a specific role in controlling the natural bacterial flora of the organism. Analogous synthetic peptides with high homology to Cecropin B from *H. cecropia*, also exhibited *in vitro* toxicity for a number of plant pathogenic bacteria at the micromolar level [Nordeen *et al.*, 1992]. No toxicity was found against fungi or cells derived from eukaryotes [Steiner *et al.*, 1981; Wade *et al.*, 1990]. These studies indicated that cecropins exhibit toxicity mainly to bacteria.

1.3.5 Mechanism of action

Most studies undertaken to elucidate the mechanism of action of cecropins were performed with the cecropins from *H. cecropia*. Therefore, in the following, 'cecropins' without further identification will be those from *H. cecropia*, unless otherwise stated.

^{1}H NMR indicated that the largest part of Cecropin A and B are in helical conformation in a hydrophobic environment [Holak *et al.*, 1988]. The first amphipathic α-helix of these cecropins exceeds 20 residues and hence could form a membrane-spanning α-helix [Lear *et al.*, 1988]. Upon oligomerization of such amphipathic α-helices in the membrane, these can ultimately form pores by segregating the hydrophobic and hydrophilic parts of the helices away and towards the central axis of the pore, respectively [reviewed in Ojcius and Young, 1991]. The formation of such pores by Cecropin A, B and D was confirmed in 1988 by Christensen *et al.* [1988], who showed that Cecropin A and B formed voltage dependent ion channels, with an approximate diameter of 4 nm in a planar lipid membrane. On the contrary, Cecropin D produced voltage-independent small channels. The presence of the flexible hinge between the amphipathic amino-terminal α-helix and the more hydrophobic carboxy-terminal α-helix, was crucial for the voltage dependent nature of the channel. The presence of cholesterol in the bilayer reduced the conductances caused by cecropins. This is in agreement with previous findings that cecropins only act on bacteria, which have membranes lacking cholesterol. Artificial liposomes were also lysed by Cecropin A indicating that the phospholipid bilayer is the target of cecropin activity [Steiner *et al*, 1988]. The reported ionophore activity of Sarcotoxin I from *S. peregrina*, apparent from K^+ leakage from and ATP depletion of *Escherichia coli* cells after treatment with the peptide [Okada and Natori, 1985a] is also in agreement with pore formation in biological membranes by cecropins.

Cecropins are present as random coil structures in aqueous solution [Steiner, 1982], and most probably adopt their biologically active structure after interacting with a bacteriological membrane [Holak *et al.*, 1988]. Some of the results of experiments, in which substitutions, additions and deletions of amino acids in Cecropin A, B and D peptides were tested for toxicity [Andreu *et al.*, 1992; Boman *et al.*, 1989; Fink *et al.*, 1989; Samakovlis *et al.*,

1990; Steiner *et al.*, 1981; Van Hofsten *et al.*, 1985; Wade *et al.*, 1990], will be discussed below together with the most likely model for interaction of cecropins with bacteriological membranes.

When the aromatic residue at position 2 of Cecropin A (Trp; see Figure 4) is removed or substituted by a non-aromatic residue (Glu), toxicity is lost [Andreu *et al.*, 1983, 1985]. Conversely, toxicity is equivalent when the Trp at position 2 of Cecropin A is substituted for by Phe, another aromatic residue. This indicates that an aromatic residue at position 2 is essential for toxicity. This residue is most likely involved in initiating the interaction between Cecropin A (in random coil) and the bacteriological membrane, and the formation of the helices [Holak *et al.*, 1988]. The introduction of helix-breaking residues (Pro) at position 4 and 8 of Cecropin A drastically reduced toxicity, indicating the essence of the first helix for toxicity [Andreu *et al.*, 1985; Steiner *et al.*, 1988]. Deletion of the four carboxy-terminal amino acids only slightly affected toxicity and hence these are probably of minor importance for toxicity [Merrifield *et al.*, 1982]. Toxicity for bacteria was identical for the D- and (naturally occurring) L-enantiomers of Cecropin A [Wade *et al.*, 1990]. In addition, both formed the same kind and number of ion channels with the same electrical conductances. This indicates that no specific interactions with a receptor are required for exposing toxicity.

In addition to synthetic cecropin counterparts, resembling the natural peptides derived from insect hemolymph, cecropin precursors and chimaeric cecropins have been synthesized and tested for toxicity. A synthetic Cecropin A peptide with a carboxy-terminal glycine residue instead of an amide group, was almost as toxic for four bacterial species as the amidated counterpart [Boman *et al.*, 1989a]. Amidation of the carboxy-terminal amino acid is hence not essential for toxicity. The Cecropin A and B precursors, containing two or four amino acids preceding the first amino acid of the mature peptide, which are subsequently removed by DPAP, were less toxic. This suggests that processing by DPAP is essential for full toxicity. Shorter amphipathic peptides did not form ion channels in contrast to full-length Cecropin A, B and D, and the flexible hinge present in the natural cecropins (see Figure 4) was required for the observation of a time-variant, voltage-dependent conductance [Christensen *et al.*, 1988]. However, the shorter peptides did bind to the bacterial membrane. These experiments led to the following mechanism of interaction between the cecropins and bacterial membranes.

The first step is an electrostatic interaction between cecropins and the bilayer-water interface. The next step could be the insertion of the more hydrophobic carboxy-terminal part into the hydrophobic membrane core, which only happens for those peptides that have a flexible hinge between the amphipathic helix and the carboxyl-terminal segment. Simultaneously, the amphipathic α-helix is formed. The third step most likely is the insertion of this positively charged amphipathic α-helix into the lipid bilayer. Upon oligomerization this results in membrane-spanning helices with the charged residues forming the water-filled pore.

Recently, hybrids of Cecropin A and mellitin, a bee venom toxin, have been synthesized and tested for antibacterial action and the formation of voltage-dependent ion channels [Andreu *et al.*, 1992; Boman *et al.*, 1989b; Wade *et al.*, 1990]. Certain hybrids appeared to be more toxic for bacteria than Cecropin A itself [Boman *et al.*, 1989b; Wade *et al.*, 1990]. However, even the 15-residue hybrids between Cecropin A and mellitin, which were designed to adapt an amphipathic α-helix, were as toxic for bacteria as full-length Cecropin A, suggesting a different mechanism for antibacterial action as described above. The adaptation of a 3_{10} helix instead of an α-helix, or the association of monomers to give multimers which are capable of spanning a membrane, have been suggested [Andreu *et al.*, 1992]. The exact mechanism by which these hybrids exhibit antibacterial activity however, remains to be investigated.

1.3.6 Biological role

The toxicity spectra of cecropins and the induction of cecropin gene expression by infection, in hemocytes and other defense-related organelles (see § 1.3.4), together with the transient nature of expression (§ 1.3.2), suggest that cecropins have a role in the control of bacterial infections. A similar role in controlling bacterial growth in the intestine is supposed for the mammalian Cecropin P1 [Lee *et al.*, 1989].

Insect pathogenic bacteria have been reported to be less susceptible towards cecropin activity. One example of such an insect-pathogenic bacterium is *Bacillus thuringiensis*. This bacterium is capable of infecting the silkmoth by evading toxicity of cecropins and attacins through the secretion of a protease that specifically degrades these defense-related peptides [Dalhammar and Steiner, 1984]. For this reason, this protease was called immune inhibitor A (InA). Molecular characterization of InA indicated that it is a metalloprotease, with homology to thermolysin and elastase around the active site [Lövgren *et al.*, 1990]. InA probably attacks the hydrophobic residues of cecropins (and attacins), which are exposed due to the open (random-coil) structure of cecropins in aqueous solution [Dalhammar and Steiner, 1984].

1.3.7 Concluding remarks and areas for further research

In conclusion, cecropins are a family of basic peptides, showing overall structural homology. In contrast to the thionins, cecropins are present as random-coil structures in aqueous solutions. They probably fold into their biologically active structure upon interaction with the bacterial membrane. Similarly to the mechanism of action by which thionins exhibit toxicity, an electrostatic interaction is the first step. Upon interaction of the second-half of cecropins with the membrane, the amphipathic α-helix is formed. This helix subsequently inserts into the lipid membrane, resulting in a membrane-spanning helix. Upon oligomerization of a number of these helices, pores are formed. Pore-formation is not dependent on a membrane receptor, and is markedly inhibited in membranes containing cholesterol (a characteristic of eukaryotic membranes). Toxicity results from leakage of cellular constituents through the pores. Cecropins have a role in controlling the bacterial flora of insects.

Their toxicity for a large number of different bacterial species, renders these peptides into potential tools for engineering resistance into plants. To prove this potential, expression studies in transgenic plants are a powerful tool.

Acknowledgements

The author wishes to thank Dirk Bosch, Ab van Kammen, Jan-Peter Nap, Andy Pereira and Willem Stiekema for critical reading of the manuscript and helpful suggestions.

1.4 References

Andresen I, Becker W, Schlüter K, Burges J, Parthier B, Apel K: The identification of leaf thionin as one of the main jasmonate-induced proteins of barley (*Hordeum vulgare*). Plant Mol Biol 19: 193-204 (1992).

Andreu D, Merrifield RB, Steiner H, Boman HG: N-terminal analogues of cecropin A: synthesis, antibacterial activity, and conformational properties. Biochem 24: 1683-1688 (1985).

Andreu D, Merrifield RB, Steiner H, Boman HG: Solid-phase synthesis of cecropin A and related peptides. Proc Natl Acad Sci USA 80: 6475-6479 (1983).

Andreu D, Ubach J, Boman A, Wåhlin B, Wade D, Merrifield RB, Boman HG: Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity. FEBS Lett 296: 190-194 (1992).

Angerhofer CK, Shier WT, Vernon LP: Phospholipase activation in the cytotoxic mechanism of thionin purified from nuts of *Pyrularia pubera*. Toxicon 28: 547-557 (1990).

Apel K, Bohlmann H, Reimann-Philipp U: Leaf thionins, a novel class of putative defense factors. Physiol Plant 80: 315-321 (1990).

Auguste P, Hugues M, Mourre C, Moinier D, Tartar A, Lazdunski M: Scyllatoxin, a blocker of Ca^{2+}-activated K^+ channels: structure-function relationships and brain localization of the binding sites. Biochem 31: 648-654 (1992).

Bairoch A: PROSITE: a dictionary of protein sites and patterns. University of Geneva, first edition (1989).

Bairoch A: PROSITE: a dictionary of protein sites and patterns. University of Geneva, first update (1990).

Balls AK, Hale WS: A sulphur-bearing constituent of the petroleum ether extract of wheat flour (preliminary report). Cereal Chem 17: 243-245 (1940).

Balls AK, Hale WS, Harris TH: A crystalline protein obtained from a lipoprotein of wheat flour. Cereal Chem 19: 279-288 (1942).

Baute R, Richir C: Sur la sensibilité à la purothionine d'une souche d'*Aspergillus flavus*. C R Seances Soc Biol Ses Fil 160:1567-1570 (1966).

Békés F: A study of purothionin isolated from the petroleum ether extract of wheat flour. Acta Aliment 6: 39-57 (1975).

Békés F, Lásztity R: Isolation and determination of amino acid sequence of avenothionin, a new purothionin analogue from oat. Cereal Chem 58: 360-361 (1981).

Békés F, Smied I: Assay into the protein-lipid complexes of wheat flour soluble in petroleum ether. Acta Aliment 10: 229-253 (1981).

Békés F, Smied I, Lásztity R: Investigation of thionins of wheat, barley, rye and oat. Dev Food Sci 5: 163-168 (1982).

Békés F: Study of purothionin analogues of certain cereals. Acta Aliment 10: 343-356 (1981).

Bohlmann H, Apel K: Isolation and characterization of cDNA's coding for leaf-specific thionins closely related to the endosperm-specific hordothionin of barley (*Hordeum vulgare* L.). Mol Gen Genet 207: 446-454 (1987).

Bohlmann H, Apel K: Thionins. Annu Rev Plant Physiol Plant Mol Biol 42: 227-240 (1991).

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 defence mechanism of plants. EMBO J 7: 1559-1565 (1988).

Boman HG: Antibacterial peptides: key components needed in immunity. Cell 65: 205-207 (1991).

Boman HG, Boman IA, Andreu A, Li Z-q, Merrifield RB, Schlenstedt G, Zimmermann R: Chemical synthesis and enzymic processing of precursor forms of cecropins A and B. J Biol Chem 264: 5852-5860 (1989a).

Boman HG, Hultmark D: Cell-free immunity in insects. Annu Rev Microbiol 41: 103-126 (1987).

Boman HG, Wade D, Boman IA, Wåhlin B, Merrifield RB: Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids. FEBS Lett 259: 103-106 (1989b).

Cammue BPA, De Bolle MFC, Terras FRG, Proost P, Van Damme J, Rees SB, Vanderleyden J, Broekaert WF: Isolation and characterization of a novel class of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. J Biol Chem 267: 2228-2233 (1992).

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).

Carmona MJ, Hernández-Lucas C, San Martin C, González P, García-Olmedo F: Subcellular localization of type I thionins in the endosperms of wheat and barley. Protoplasma: in press (1993).

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 (1981).

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).

Christensen B, Fink J, Merrifield RB, Mauzerall D: Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. Proc Natl Acad Sci USA 85: 5072-5076 (1988).

Clore GM, Nilges M, Sukumaran DK, Brünger AT, Karplus M, Gronenborn AM: The three-dimensional structure of α1-purothionin in solution: combined use of nuclear magnetic resonance, distance geometry and restrained molecular dynamics. EMBO J 5: 2729-2735 (1986).

Clore GM, Sukumaran DK, Gronenborn AM, Teeter MM, Whitlow M, Jones BL: Nuclear magnetic resonance study of the solution structure of α1-purothionin. Sequential resonance assignment, secondary structure and low resolution tertiary structure. J Mol Biol 193: 571-578 (1987).

Colilla FJ, Rocher A, Mendez E: γ-purothionins: amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm. FEBS Lett 270: 191-194 (1990).

Coulson EJ, Harris TH, Axelrod B: Effect on small laboratory animals of the injection of the crystalline hydrochloride of a sulfur protein from wheat flour. Cereal Chem 19: 301-307 (1941).

Dalhammar G, Steiner H: Characterization of inhibitor A, a protease from *Bacillus thuringiensis* which degrades attacins and cecropins, two classes of antibacterial proteins in insects. Eur J Biochem 139: 247-252 (1984).

De Weille JR, Schweitz H, Maes P, Tartar A, Lazdunski M: Calciseptine, a peptide isolated from black mamba venom, is a specific blocker of the L-type calcium channel. Proc Natl Acad Sci USA 88: 2437-2440 (1991).

Dickinson L, Russell V, Dunn PE: A family of bacteria-regulated, cecropin D-like peptides from *Manduca sexta*. J Biol Chem 263: 19424-19429 (1988).

Ebrahim-Nesbat F, Behnke S, Kleinhofs A, Apel K: Cultivar-related differences in the distribution of cell-wall-bound thionins in compatible and incompatible interactions between barley and powdery mildew. Planta 179: 203-210 (1989).

Engström Å, Engström P, Tao Z-j, Carlsson A, Bennich H: Insect immunity. The primary structure of the antibacterial protein attacin F and its relation to two native attacins from *Hyalophora cecropia*. EMBO J 3: 2065-2070 (1984a).

Engström Å, Xanthopoulos KG, Boman HG, Bennich H: Amino acid and cDNA sequences of lysozyme from *Hyalophora cecropia*. EMBO J 4: 2119-2122 (1985).

Engström P, Carlsson A, Engström Å, Tao Z-j, Bennich H: The antibacterial effect of attacins from the silkmoth *Hyalophora cecropia* is directed against the outer membrane of *Escherichia coli*. EMBO J 3: 3347-3351 (1984b).

Evans J, Wang Y, Shaw K-P, Vernon LP: Cellular responses to *Pyrularia* thionin are mediated by Ca^{2+} influx and phospholipase A_2 activation and are inhibited by thionin tyrosine iodination. Proc Natl Acad Sci USA 86: 5849-5853 (1989).

Evett GE, Donaldson DM, Vernon LP: Biological properties of *Pyrularia* thionin prepared from nuts of *Pyrularia pubera*. Toxicon 24: 622-625 (1986).

Faye I, Wyatt GR: The synthesis of antibacterial proteins in isolated fat body from cecropia silkmoth pupae. Experientia 36: 1325-1326 (1980).

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).

Fernandez de Caleya R, Hernandez-Lucas C, Carbonero P, García-Olmedo F: Gene expression in alloploids: genetic control of lipopurothionins in wheat. Genetics 83: 687-699 (1976).

Fincher GB: Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. Annu Rev Plant Physiol Plant Mol Biol 40: 305-340 (1989).

Fink J, Merrifield RB, Boman A, Boman HG: The chemical synthesis of cecropin D and an analog with enhanced antibacterial activity. J Biol Chem 264: 6260-6267 (1989).

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).

Fontecilla-Camps JC, Almassy RJ, Ealick SE, Watt DD, Feldmann RJ, Bugg CE: Architecture of scorpion neurotoxins: a class of membrane-binding proteins. TIBS 6: 291-296 (1981).

García-Olmedo F, Carbonero P, Hernández-Lucas C, Paz-Ares J, Ponz F, Vicente O, Sierra JM: Inhibition of eukaryotic cell-free protein synthesis by thionins from wheat endosperm. Biochim Biophys Acta 740: 52-56 (1983).

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), Genes involved in plant defense, pp. 283-302. Springer-Verlag, Wien (1992).

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).

Gausing K: Thionin genes specifically expressed in barley leaves. Planta 171: 241-246 (1987).

Gudmundsson GH, Lidholm D-A, Åsling B, Gan R, Boman HG: The cecropin locus. Cloning and expression of a gene cluster encoding three antibacterial peptides in *Hyalophora cecropia*. J Biol Chem 266: 11510-11517 (1991).

Gu Q, Kawata EE, Morse M-J, Wu H-M, Cheung AY: A flower-specific cDNA encoding a novel thionin in tobacco. Mol Gen Genet 234: 89-96 (1992).

Hase T, Matsubara H, Yoshizumi H: Disulfide bonds of purothionin, a lethal toxin for yeasts. J Biochem 83: 1671-1678 (1978).

Hendrickson WA, Teeter MM: Structure of the hydrophobic protein crambin determined directly from the anomalous scattering of sulphur. Nature 290: 107-112 (1981).

Hernández-Lucas C, Fernandez de Caleya R, Carbonero P: Inhibition of brewer's yeasts by wheat purothionins. Appl Microbiol 28: 165-168 (1974).

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).

Holak TA, Engström Å, Kraulis PJ, Lindeberg G, Bennich H, Alwyn Jones T, Gronenborn AM, Clore GM: The solution conformation of the antibacterial peptide cecropin A: a nuclear magnetic resonance and dynamical simulated annealing study. Biochem 27: 7620-7629 (1988).

Hultmark D, Engström Å, Andersson K, Steiner H, Bennich H, Boman HG: Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*. EMBO J 2: 571-576 (1983).

Hultmark D, Engström Å, Bennich H, Kapur R, Boman HG: Insect immunity: Isolation and structure of cecropin D and four minor antibacterial components from cecropia pupae. Eur J Biochem 127: 207-217 (1982).

Hultmark D: Immune reactions in Drosophila and other insects: a model for innate immunity. TIG 9: 178-183 (1993).

Hultmark D, Steiner H, Rasmusson T, Boman HG: Insect immunity. Purification and properties of three inducible bactericidal protein from hemolymph of immunized pupae of *Hyalophora cecropia*. Eur J Biochem 106: 7-16 (1980).

Johannes E, Brosnan JM, Sanders D: Calcium channels and signal transduction in plant cells. BioEssays 13: 331-336 (1991).

Johnson TC, Wada K, Buchanan BB, Holmgren A: Reduction of purothionin by the wheat seed thioredoxin system. Plant Physiol 85: 446-451 (1987).

Jones BL, Lookhart GL, Johnson DE: Improved separation and toxicity analysis methods for purothionins. Cereal Chem 62: 327-331 (1985).

Jones BL, Mak AS: Amino acid sequences of the two α-purothionins of hexaploid wheat. Cereal Chem 54: 511-523 (1976).

Jones BL, Meredith P: Inactivation of alpha-amylase activity by purothionins. Cereal Chem 59: 321 (1982).

Judd AM, Vernon LP, MacLeod RM: Pyrularia thionin increases arachidonate liberation and prolactin and growth hormone release from anterior pituitary cells. Toxicon 30: 1563-1573 (1992).

Kashimoto T, Sakakibara R, Huynh QK, Wada H, Yoshizumi H: The effect of purothionin on bovine adrenal medullary cells. Res Comm Chem Pathol Pharmacol 26: 221-224 (1979).

Kato Y, Taniai K, Hirochika H, Yamakawa M: Expression and characterization of cDNA's for cecropin B, an antibacterial protein of the silkworm, *Bombyx mori*. Insect Biochem Molec Biol 23: 285-290 (1993).

Kockum K, Faye I, Van Hofsten P, Lee J-Y, Xanthopoulos KG, Boman HG: Insect immunity. Isolation and sequence of two cDNA clones corresponding to acidic and basic attacins from *Hyalophora cecropia*. EMBO J 3: 2071-2075 (1984).

Konopa J, Woynarowski JM, Lewandowska-Gumieniak M: Isolation of viscotoxins. Cytotoxic basic polypeptides from *Viscum album* L. Hoppe-Seyler's Z Physiol Chem 361: 1525-1533 (1980).

Kramer KJ, Klassen LW, Jones BL, Speirs RD, Kammer AE: Toxicity of purothionin and its homologues to the tobacco hornworm, *Manduca sexta* (L.) (*Lepidoptera*: *Sphingidae*). Toxicol Appl Pharmacol 48: 179-183 (1979).

Kylsten P, Samakovlis C, Hultmark D: the cecropin locus in *Drosophila*; a compact gene cluster involved in the response to infection. EMBO J 9: 217-224 (1990).

Lear JD, Wasserman ZR, DeGrado WF: Synthetic amphiphilic peptide models for protein ion channels. Science 240: 1177-1181 (1988).

Lecomte JTJ, Jones BL, Llinás M: Proton magnetic resonance studies of barley and wheat thionins: structural homology with crambin. Biochem 21: 4843-4849 (1982).

Lecomte JTJ, Kaplan D, Llinás M, Thunberg E, Samuelsson G: Proton magnetic resonance characterization of phoratoxins and homologous proteins related to Crambin. Biochem 26: 1187-1194 (1987).

Lee J-Y, Boman A, Chuanxin S, Andersson M, Jörnvall H, Mutt V, Boman HG: Antibacterial peptides from pig intestine: isolation of a mammalian cecropin. Proc Natl Acad Sci USA 86: 9159-9162 (1989).

Lidholm D-A, Gudmundsson GH, Xanthopoulos KG, Boman HG: Insect immunity: cDNA clones coding for the precursor forms of cecropins A and D, antibacterial proteins from *Hyalophora cecropia*. FEBS Lett 226: 8-12 (1987).

Lövgren A, Zhang M, Engström A, Dalhammar G, Landén R: Molecular characterization of immune inhibitor A, a secreted virulence protease from *Bacillus thuringiensis*. Mol Microbiol 4: 2137-2146 (1990).

Mak AS, Jones BL: The amino acid sequence of wheat ß-purothionin. Can J Biochem 22: 835-842 (1976).

Matsumoto N, Okada M, Takahashi H, Ming QX, Nakajima Y, Nakanishi Y, Komano H, Natori S: Molecular cloning of a cDNA and assignment of the C-terminal of sarcotoxin IA, a potent antibacterial protein of *Sarcophaga peregrina*. Biochem J 239: 717-722 (1986).

Mellstrand ST, Samuelsson G: Phoratoxin, a toxic protein from the mistletoe *Phoradendron tomentosum* subsp. *macrophyllum* (*Loranthaceae*). Improvements in the isolation procedure and further studies on the properties. Eur J Biochem 32: 143-147 (1973).

Mellstrand ST, Samuelsson G: Phoratoxin, a toxic protein from the mistletoe *Phoradendron tomentosum* subsp. *macrophyllum* (*Loranthaceae*). The amino acid sequence. Acta Pharm Suec 11: 347-360 (1974a).

Mellstrand ST, Samuelsson G: Phoratoxin, a toxic protein from the mistletoe *Phoradendron tomentosum* subsp. *macrophyllum* (*Loranthaceae*). The disulphide bonds. Acta Pharm Suec 11: 367-374 (1974b).

Mendez E, Moreno A, Colilla F, Pelaez F, Limas GG, Mendez R, Soriano F, Salinas M, De Haro C: Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin, γ-hordothionin, from barley endosperm. Eur J Biochem 194: 533-539 (1990).

Merrifield RB, Vizioli LD, Boman HG: Synthesis of the antibacterial peptide cecropin A(1-33). Biochem 21: 5020-5031 (1982).

Morishima I, Suginaka S, Ueno T, Hirano H: Isolation and structure of cecropins, inducible antibacterial peptides, from the silkworm, *Bombyx mori*. Comp Biochem Physiol 95B: 551-554 (1990).

Nakanishi T, Yoshizumi H, Tahara S, Hakura A, Toyoshima K: Cytotoxicity of purothionin-A on various animal cells. Gann 70: 323-326 (1979).

Nordeen RO, Sinden SL, Jaynes JM, Owens LD: Activity of cecropin SB37 against protoplasts from several plant species and their bacterial pathogens. Plant Sci 82: 101-107 (1992).

Ohtani S, Okada T, Kagamiyama H, Yoshizumi H: The amino acid sequence of purothionin A, a lethal toxic protein for brewer's yeasts from wheat. Agric Biol Chem 39: 2269-2270 (1975).

Ohtani S, Okada T, Yoshizumi H, Kagamiyama H: Complete primary structures of two subunits of purothionin A, a lethal protein for brewer's yeast from wheat flour. J Biochem 82: 753-767 (1977).

Ojcius DM, Young D-E: Cytolytic pore-forming proteins and peptides: is there a common structural motif? TIBS 16: 225-229 (1991).

Okada M, Natori S: Ionophore activity of sarcotoxin I, a bactericidal protein of Sarcophaga peregrina. Biochem J 229: 453-458 (1985a).

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).

Okada M, Natori S: Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae. J Biol Chem 260: 7174-7177 (1985b).

Olivera BM, Gray WR, Zeikus R, McIntosh JM, Varga J, Rivier J, De Santos V, Cruz LJ: Peptide neurotoxins from fish-hunting cone snails. Science 230: 1338-1343 (1985).

Olson T, Samuelsson G: The amino acid sequence of Viscotoxin A2 from the European mistletoe (*Viscum album* L., *Loranthaceae*). Acta Chem Scand 26: 585-595 (1972).

Osorio e Castro VR, Ashwood ER, Wood SG, Vernon LP: Hemolysis of erythrocytes and fluorescence polarization changes elicited by peptide toxins, aliphatic alcohols, related glycols and benzylidene derivatives. Biochim Biophys Acta 1029: 252-258 (1990).

Osorio e Castro VR, Van Kuiken BA, Vernon LP: Action of a thionin isolated from nuts of *Pyrularia pubera* on human erythrocytes. Toxicon 27: 501-510 (1989).

Osorio e Castro VR, Vernon LP: Hemolytic activity of thionin from *Pyrularia pubera* nuts and snake venom toxins of *Naja naja* species: *Pyrularia* thionin and snake venom cardiotoxin compete for the same membrane site. Toxicon 27: 511-517 (1989).

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).

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).

Qu X-m, Steiner H, Engström Å, Bennich H, Boman HG: Insect immunity: isolation and structure of cecropins B and D from pupae of the Chinese oak silk moth, *Antheraea pernyi*. Eur J Biochem 127: 219-224 (1982).

Ranjeva R, Thuleau P, Schroeder JI: Signal transduction and calcium channels in higher plants. Curr Opin Biotechn 4: 172-176 (1993).

Rasmussen SK, Rasmussen CE: Full length cDNA encoding barley toxin ß-hordothionin. Plant Mol Biol 21: 579 (1993).

Redman DG, Fisher N: Purothionin analogues from barley flour. J Sci Food Agric 20: 427-432 (1968).
Reimann-Philipp U, Behnke S, Batschauer A, Schäfer E, Apel K: The effect of light on the biosynthesis of leaf-specific thionins in barley. *Hordeum vulgare*. Eur J Biochem 182: 283-289 (1989a).
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 (1989b).
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).
Rosell S, Samuelsson G: Effect of mistletoe viscotoxin and phoratoxin on blood circulation. Toxicon 4: 107-110 (1966).
Rosetto M, Manetti AGO, Marchini D, Dallai R, Telford JL, Baldari CT: unpublished results, sequences submitted to the EMBL Data Library (1993).
Samakovlis C, Kimbrell DA, Kylsten P, Engström Å, Hultmark D: The immune response in *Drosophila*: pattern of cecropin expression and biological activity. EMBO J 9: 2969-2976 (1990).
Samuelsson G: Mistletoe toxins. Syst Zool 22: 566-569 (1973).
Samuelsson G, Pettersson BM: The amino acid sequence of Viscotoxin B from the European mistletoe (*Viscum album* L., *Loranthaceae*). Eur J Biochem 21: 86-89 (1971).
Samuelsson G, Pettersson B: Toxic proteins from the mistletoe *Dendrophtora clavata*. II. The amino acid sequence of denclatoxin B. Acta Pharm Suec 14: 245-254 (1977).
Schrader G, Apel K: Isolation and characterization of cDNA's encoding viscotoxins of mistletoe (*Viscum album*). Eur J Biochem 198: 549-553 (1991).
Steiner H, Andreu D, Merrifield RB: Binding and action of cecropin analogues: antibacterial peptides from insects. Biochim Biophys Acta 939: 260-266 (1988).
Steiner H, Hultmark D, Engström Å, Bennich H, Boman HG: Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292: 246-248 (1981).
Steiner H: Secondary structure of the cecropins: antibacterial peptides from the moth *Hyalophora cecropia*. FEBS Lett 137: 283-287 (1982).
Stuart LS, Harris TH: Bactericidal and fungicidal properties of a crystalline protein isolated from unbleached wheat flour. Cereal Chem 19: 288-300 (1942).
Taniai K, Kato Y, Hirochika H, Yamakawa M: Isolation and nucleotide sequence of cecropin B cDNA clones from the silkworm, *Bombyx mori*. Biochim Biophys Acta 1132: 203-206 (1992).
Teeter MM, Ma X-Q, Rao U, Whitlow M: Crystal structure of a protein-toxin α1-purothionin at 2.5 Å and a comparison with predicted models. Proteins: structure, function, and genetics 8: 118-132 (1990).
Teeter MM, Mazer JA, L'Italien JJ: Primary structure of the hydrophobic plant protein crambin. Biochem 20: 5437-5443 (1981).
Teeter MM, Whitlow M: Test of circular dichroism (CD) methods for crambin and CD-assisted secondary structure prediction of its homologous toxins. Proteins 4: 262-273 (1988).
Teshima T, Ueki, Y, Nakai T, Shiba T, Kikuchi M: Structure determination of lepidopteran, self-defense substance produced by silkworm. Tetrahedron 42: 829-834 (1986).
Thunberg E, Samuelsson G: The amino acid sequence of ligatoxin A, from the mistletoe *Phoradendron liga*. Acta Pharm Suec 19: 447-456 (1982).
Trewavas A, Gilroy S: Signal transduction in plant cells. TIG 7: 356-361 (1991).
Tryselius Y, Samakovlis C, Kimbrell DA, Hultmark D: *CecC*, a cecropin gene expressed during metamorphosis in *Drosophila* pupae. Eur J Biochem 204: 385-399 (1992).
Van Etten CH, Nielsen HC, Peters JE: A crystalline polypeptide from the seed of *Crambe abyssinica*. Phytochem 4: 467-473 (1965).
Van Hofsten P, Faye I, Kockum K, Lee J-Y, Xanthopoulos KG, Boman IA, Boman HG, Engström Å, Andreu D, Merrifield RB: Molecular cloning, cDNA sequencing, and chemical synthesis of cecropin B from *Hyalophora cecropia*. Proc Natl Acad Sci USA 82: 2240-2243 (1985).
Vermeulen JAWH, Lamerichs RMJN, Berliner LJ, De Marco A, Llinás M, Boelens R, Alleman J, Kaptein R: ^{1}H NMR characterization of two crambin species. FEBS Lett 219: 426-430 (1987).
Vernon LP, Evett GE, Zeikus RD, Gray WR: A toxic thionin from *Pyrularia pubera*: purification, properties, and amino acid sequence. Arch Biochem Biophys 238: 18-29 (1985).
Vernon LP, Rogers A: Binding properties of *Pyrularia* thionin and *Naja naja kaouthia* cardiotoxin to human and animal erythrocytes and to murine P388 cells. Toxicon 30: 711-721 (1992a).
Vernon LP, Rogers A: Effect of calcium and phosphate ions on hemolysis induced by *Pyrularia* thionin and *Naja naja kaouthia* cardiotoxin. Toxicon 30: 701-709 (1992b).

Wada K, Buchanan BB: Purothionins: a seed protein with thioredoxin activity. FEBS Lett 124: 237-240 (1981).
Wada K, Ozaki Y, Matsubara H, Yoshizumi H: Studies on purothionin by chemical modifications. J Biochem 91: 257-263 (1982).
Wade D, Boman A, Wåhlin B, Drain CM, Andreu D, Boman HG, Merrifield RB: All-D amino acid-containing channel-forming peptides. Proc Natl Acad Sci USA 87: 4761-4765 (1990).
Wallace BA, Kohl N, Teeter MM: Crambin in phospholipid vesicles: circular dichroism analysis of crystal structure relevance. Proc Natl Acad Sci USA 81: 1406-1410 (1984).
Whitlow M, Teeter MM: Energy minimization for tertiary structure prediction of homologous proteins: α1-purothionin and Viscotoxin A3 models from Crambin. J Biomol Struct Dynam 2: 831-848 (1985).
Winterfeld VK, Bijl LH: Viscotoxin, ein neuer Inhaltsstoff der Mistel (*Viscum album* L.). Liebigs Ann Chem 561: 107-115 (1949).
Woodward SR, Cruz LJ, Olivera BM, Hillyard DR: Constant and hypervariable regions in conotoxin propeptides. EMBO J 9: 1015-1020 (1990).
Wooley DW, Krampitz LO: Reversal by phosphatides of the antimicrobial action of a crystalline protein from wheat. J Biol Chem 146: 273-274 (1942).
Woynarowski JM, Konopa J: Interaction between DNA and viscotoxins. Hoppe-Seyler's Z Physiol Chem 361: 1535-1545 (1980).

CHAPTER 2

Analysis of the toxicity of purothionins and hordothionins for plant pathogenic bacteria

Netherlands Journal of Plant Pathology 99: 259-268 (1993)

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2.1 Abstract

Purothionins (PTHs) and hordothionins (HTHs) were purified by cation-exchange chromatography from petroleum-ether extracts of wheat and barley flour respectively. The HTHs could be separated into two fractions, HTH-1 and HTH-2. Radial diffusion assays and micro-plate broth dilution assays with a number of plant pathogenic bacteria showed that these proteins were toxic for *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker on tomato, *C. m.* subsp. *sepedonicus*, the causal agent of ring rot on potato, and *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of a spot disease on tomato and pepper. Only minor differences in toxicity between PTHs and HTHs, and between HTH-1 and HTH-2, were detected. Minor differences in toxicity of these thionins were also detected for different strains of these bacteria. The use of these plant proteins for engineering bacterial disease resistance into solanaceous crops will be discussed.

2.2 Introduction

Bacterial diseases can cause a drastic decrease of yield in certain crops. Due to bacterial diseases, the losses of the worldwide potato production can be as high as twenty-five percent [Sawyer, 1984]. Breeding for bacterial disease resistance therefore is of utmost necessity, but for most plant-bacterial pathogen combinations, traditional plant breeding has not been very successful in this respect. A different approach to control bacterial diseases in plants is offered by genetic engineering. The first genetically engineered plants which showed resistance to bacterial infection, were reported in 1989. Expression of a tabtoxin-resistant acetyltransferase in tobacco plants conferred resistance to *Pseudomonas syringae* pv. *tabaci* [Anzai *et al.*, 1989]. More recently, another example of this so-called "pathogen-derived resistance" was reported. Expression of a toxin-resistant target enzyme in sensitive plants resulted in insensitivity towards the toxin and resistance to bacterial infection [De la Fuente-Martínez *et al.*, 1992].

Another approach to engineer resistance in plants against bacteria, is the introduction and expression of proteins that have antibacterial properties. A family of genes of potential significance in this respect, are the genes coding for the so-called thionins [García-Olmedo *et al.*, 1989; Florack *et al.*, 1990]. These are low-molecular-weight ($M_r \approx 5000$) plant proteins which almost all exhibit antimicrobial activity *in vitro*. Thionins can be divided into five different types, based on the plant species and tissues in which they occur, the overall net-charge, the number of amino acids and disulphide bonds and the homology at the amino acid level [García-Olmedo *et al.*, 1989; Castagnaro *et al.*, 1992]. The best studied are the highly basic type 1 thionins, which are abundantly present in the endosperm of most Poaceae [reviewed in García-Olmedo *et al.*, 1989]. These thionins all consist of 45 amino acids, eight of which are involved in four disulphide bonds. Thionins of this class share 85% homology at the amino acid level. Type 2 thionins have been identified in leaves of the parasitic dicotyledonous plant *Pyrularia pubera* and in leaves of barley (*Hordeum vulgare*). These thionins consist of 47 or 46 amino acids respectively, are less basic and also have four disulphide bonds. Type 3 thionins have been identified in leaves and stems of a number of mistletoe species (*Viscum album*, *Phoradendron* spp. and *Dendrophtora clavata*) and consist of 46 amino acids with three disulphide bonds and are as basic as the type 2 thionins. Type 4 thionins have been identified in seeds of the Abyssinian cabbage (*Crambe abyssinica*) and consist of 46 amino acids with three disulphide bonds and are neutral in charge. Type 5 thionins are also present in the endosperm of monocotyledons, but are neutral in charge, only consist of 36 amino acids, probably have two disulphide bonds and are most likely derived

from type 1 thionins [Castagnaro *et al.*, 1992].

In vitro toxicity of the type 1 purothionins (PTHs) from wheat endosperm for a number of plant pathogenic bacteria [Fernandez de Caleya *et al.*, 1972], and more recently, toxicity of the type 1 and 2 hordothionins (HTHs) from barley for two plant pathogenic fungi [Bohlmann *et al.*, 1988] have been reported. The type 2 thionins might be directly involved in plant defence, since infection of barley with spores of powdery mildew, *Erysiphe graminis* f.sp. *hordei*, resulted in a transient increase of type 2-thionin mRNA levels in leaves [Bohlmann *et al.*, 1988]. In addition, cultivar-related differences in distribution of these type 2 thionins in epidermal cells were demonstrated in compatible and incompatible interactions between barley and powdery mildew [Ebrahim-Nesbat *et al.*, 1989].

Our objective was to study the feasibility of using the type 1 HTHs for engineering bacterial disease resistance into a number of different plant species, especially into solanaceous crops. To this end, the toxicity of the HTHs for a number of bacterial pathogens causing serious damage on these plants, was determined first and compared with the toxicity of the PTHs, which were reported previously to inhibit growth of certain plant pathogenic bacteria [Fernandez de Caleya *et al.*, 1972].

This study reports the purification of PTHs and HTHs from wheat and barley endosperm, and a comparative analysis of the *in vitro* toxicity of these purified thionins for a number of plant pathogenic bacteria. Possible applications of thionin encoding sequences for engineering bacterial disease resistance into crops will be outlined.

2.3 Materials and methods

2.3.1 Thionin extraction and purification.

HTHs were isolated from flour of seeds of *H. vulgare* cv. Femina, and PTHs from *Triticum aestivum* cv. Camp-Remy. Seed flour was extracted with petroleum-ether according to Békés [1975] and after concentration treated with hydrochloric acid in ethanol as described by Balls *et al.* [1942]. The "crude thionin" fraction was treated with water and ethanol as described by Fisher *et al.* [1968], to generate partially purified thionins. These were applied onto a CM Sepharose Fast Flow (Pharmacia) column and eluted with a linear gradient of 0.3 or 0.4 to 1.0 M NaCl in 50 mM phosphate buffer. Thionin containing fractions were pooled, concentrated and desalted by overnight dialysis against distilled water using Spectra/Por 6 (Spectrum) dialysis membranes (molecular weight cut-off 2000). The amount of protein was estimated by the method of Bradford [1976]. Before assaying toxicity, purified thionins were sterilized by filtration through Optex filters (0.22 μm; Millipore).

2.3.2 Electrophoresis and Western analysis.

The molecular weight of the proteins present in the pooled fractions was determined by SDS-polyacrylamide gel electrophoresis on 12.5% gels in the presence of 7 M urea (SDSurea-PAGE), as described by Swank and Munkres [1971]. Molecular weight markers were from Sigma (MWS-877P) and a synthetic cecropin B (M_r = 3832), added as an additional marker, was from American Peptide Company, Inc. (Sunnyvale, USA). The purity of the thionins was determined by electrophoresis of the samples over a 20% acidic-polyacrylamide gel (acidPAGE). The separating gel buffer contained 0.375 M HAc and 0.09 M KOH, pH 4.3. A stacking gel consisted of 4% polyacrylamide in 0.063 M HAc and 0.09 M KOH, pH 6.8. The single electrode buffer contained 0.035 M ß-alanine and 0.017 M HAc. Samples were

prepared in 0.017 M HAc, incubated at 70°C for 10 min and allowed to cool to room temperature. Prior to loading, 1/20 vol loading buffer containing 0.063 M HAc, 0.09 M KOH, 1 M sucrose and 8.25 mM Pyronin Y (Sigma) was added to each sample. Gels were run at 180 V and 60 mA for 90 min on a 2050 Midget (LKB) electrophoresis unit. Gels were stained with Coommassie Brilliant Blue R-250. Western analysis was performed according to Towbin *et al.* [1979] using an antiserum raised against the HTHs from barley endosperm (kindly provided by K. Apel, ETH Zentrum, Zürich, Switzerland) and an alkaline phosphatase-labelled secondary antibody.

2.3.3 Bacterial strains and growth media.

Toxicity of thionins was tested for the following bacteria: *Clavibacter michiganensis* subspecies (subsp.) *michiganensis*, the causal agent of bacterial canker on tomato; *C. m.* subsp. *sepedonicus*, the causal agent of ring rot on potato; *Erwinia amylovora*, the causal agent of bacterial blight on a number of plant species; *E. carotovora* subsp. *atroseptica*, the causal agent of black leg on potato; *E. c.* subsp. *carotovora* and *E. chrysanthemi*, the causal agents of soft rot diseases on potato; *E. salicis*, the causal agent of the watermark disease on willow; *Pseudomonas solanacearum*, the causal agent of bacterial wilt on a number of solanaceous crops; *P. syringae* pathovar (pv.) *tabaci*, the causal agent of wildfire disease on tobacco; *P. s.* pv. *tomato*, the causal agent of a spot disease on tomato and *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of a leaf spot disease on tomato and pepper. The strains of these plant pathogenic bacteria used in this work, and the host plants from which they were isolated are listed in Table 1, and were kindly provided by the Netherlands Plant Protection Service, Wageningen, The Netherlands.

Erwinia spp.and *Pseudomonas* spp. were grown on bouillon-agar (BA) slants containing per liter: 8 g Lab Lemco broth (Oxoid), 5 g NaCl and 15 g agar (Oxoid) at 27°C for 24 h. *C. m.* subsp. *michiganensis* and *X. c.* pv. *vesicatoria* were grown on growth-factor-agar (GFA) slants containing per liter: 0.4 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.5 g $NH_4H_2PO_4$, 1 g glucose, 3 g yeast extract (Oxoid) and 15 g agar (Oxoid) (final pH 7.2) at 27°C for 24 h. *C. m.* subsp. *sepedonicus* was grown on yeast-peptone-glucose-agar (YPGA) slants containing per liter 5 g yeast extract (Oxoid), 10 g peptone (Oxoid), 5 g D(+)-glucose and 15 g agar (Oxoid) at 20°C for 72 h. The medium used for radial diffusion assays and for counting the number of CFU was trypticase-soy-agar (TSA, Becton Dickinson). Media used for micro-plate broth dilution assays were trypticase-soy-broth (TSB, Becton Dickinson) and a nutrient-broth (NB) described by Fernandez de Caleya *et al.* [1972], containing per liter: 0.1% beef extract (Difco), 0.2% yeast extract (Oxoid), 0.5% peptone (Oxoid) and 0.5% NaCl (final pH 6.5).

2.3.4 Toxicity assays with bacteria

Inocula were prepared by resuspending bacteria from 24 to 48 h old agar slants in sterile distilled water and visual adjustment to approximately 1 x 10^8 CFU/ml using McFarland's turbidity standards [McFarland, 1907]. The actual number of CFU was determined by pour plating 100 μl of serial ten-fold dilutions in 300 μl TSA in 24-well Cluster[24] (Mark II, Costar) tissue culture plates.

Toxicity of thionins was measured by two methods. For the radial diffusion assay, 9 cm Petri dishes, containing a bacterial suspension of approximately 10^6 or 10^7 CFU in a total volume of 15 ml TSA, were used. 15 μl solutions containing 1, 5, 25 or 125 μg thionin in

water were applied to 4 mm wells, cut in the agar plate using a sterile cork borer. 15 µl solutions containing 25 µg bovine-serum-albumin (BSA) and 15 µl water were used as controls. Toxicity of thionins was measured by screening for the presence of growth inhibition zones around the wells after incubation at 27°C for 24 to 72 h, except for plates containing *C. m.* subsp. *sepedonicus* which were incubated at 20°C for 72 to 96 h. In the micro-plate broth dilution assay thionins were tested in serial, two-fold dilutions in flat-bottom 96-well microtiter-plates (Greiner). The final concentrations of thionins tested were 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 µg/ml in TSB or NB. To each well 200 µl medium was added, containing the thionin and approximately 10^6 CFU/ml of the bacterium to be tested. Plates were incubated without shaking at 27°C for 24 to 48 h, except plates containing *C. m.* subsp. *sepedonicus* which were incubated at 20°C for 72 to 96 h. After shaking of the plates for a few seconds on a micro-plate shaker, growth was measured turbidimetrically at 620 nm using an Easy Reader EAR400 AT micro-plate reader (SLT Lab. Instruments). CFU were determined as described above for the control well, for the last well with measurable bacterial growth, and for each of the wells in which no growth was observed.

2.4 Results

2.4.1 Purification of thionins

The method described here resulted in approximately 100 mg freeze-dried crude HTH and 50 mg crude PTH from 2200 g barley flour and 1200 g wheat flour, respectively. Column chromatography of the crude HTH preparation (Figure 1A) resulted in a number of fractions under two distinct peaks, which were positive for thionins, as estimated by Western analysis (Figure 2B). These were collected in two separate pools, referred to as HTH-1 (fractions no. 18 to 26) and HTH-2 (fractions no. 27 to 33). Fractions under a third peak were negative for thionins. Column chromatography of crude PTH (Figure 1B) resulted in a similar elution profile in which the fractions no. 16 to 25 appeared to be positive for thionins. These fractions were combined in a single pool and will be referred to as PTH. Concentration of pooled fractions, followed by desalting and lyophilization yielded 12.5 mg HTH-1, 34.5 mg HTH-2 and 21 mg PTH.

Sepharose column fractionation produced essentially pure thionin preparations (Figure 2). The molecular weight of the pooled fractions was approximately 5000 (Figure 2A), and Western analysis confirmed that these proteins were in fact thionins (Figure 2B). The presence of bands of molecular weight 10000, 15000 and higher, might have been caused by intermolecular disulphide bonds between thionin molecules (multimeric forms), known to occur for PTHs due to their high cystein content (Redman and Elton, 1969). The purity of the preparations was confirmed by acidPAGE (Figure 2C). Only a single band was visible in each of the preparations.

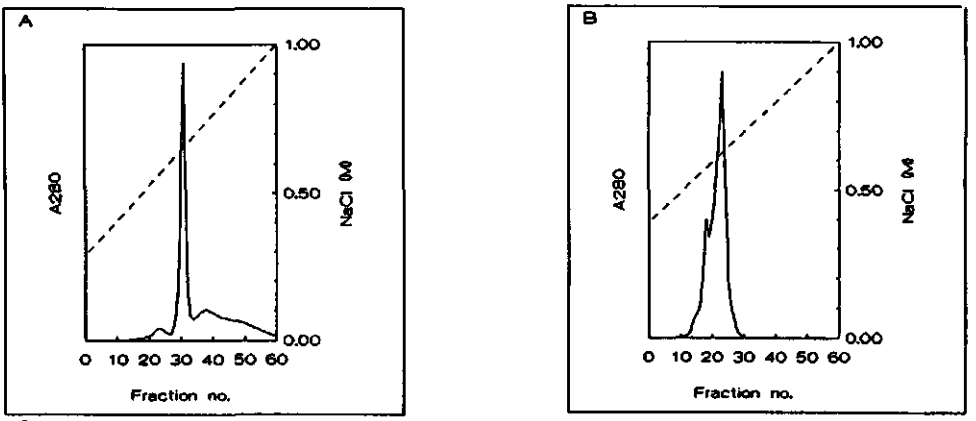


Figure 1. Elution profiles of CM Sepharose Fast Flow column of crude hordothionins (HTHs) from barley (A) and purothionins (PTHs) from wheat endosperm (B). Symbols: ———, A_{280}; ---, NaCl gradient. (A) Fractions 18 to 26 and 27 to 33 were pooled to render HTH-1 and HTH-2, respectively. (B) Fractions 16 to 25 were pooled to render PTH.

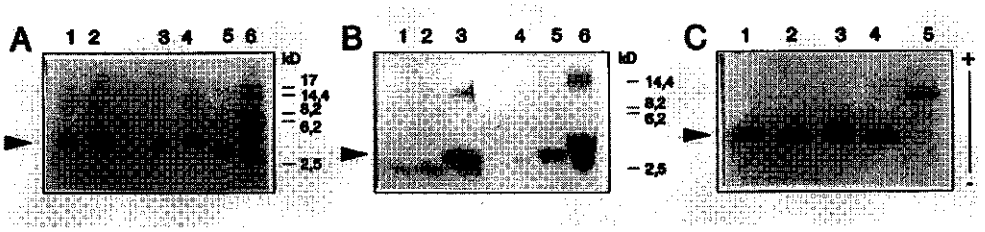


Figure 2. Molecular weight, purity and identity of pooled thionin fractions from wheat and barley endosperm. Electrophoresis patterns obtained from SDSureaPAGE (A) and acidPAGE (C), as visualized by Coommassie Brilliant Blue staining, and results from Western analysis using a hordothionin antiserum, after separation of samples by SDSureaPAGE (B). A and C: lane 1, PTH; lane 2, HTH; lane 3, HTH-1; lane 4, HTH-2; lane 5, CecropinB standard; lane 6, molecular weight marker. B: lanes 1 to 3, different amounts of HTH-1 (100, 500 and 1000 ng respectively) and lanes 4 to 6, the same amounts of HTH-2, respectively. Molecular mass standards (A & B) and the migration direction (C) are indicated on the right.

2.4.2 Toxicity assays

A fast and easy screening for toxicity of thionins to bacteria was performed on strains of nine different plant pathogenic bacteria by the radial diffusion assay. Small samples of HTH and PTH were applied to wells in bacteria-containing medium and bacterial sensitivity was measured by the presence of a growth inhibition zone around the well. *Erwinia* spp. and *Pseudomonas* spp. did not show growth inhibition zones for concentrations up to 125 μg HTH and PTH per well. However, *C. m.* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus* and *X. c.* pv. *vesicatoria* exhibited growth inhibition zones in the presence of low

concentrations of HTH or PTH (Table 1). The diameter of the growth inhibition zones increased in a dose-dependent manner and was independent of the number of bacteria plated, 10^6 or 10^7 CFU per plate, or the thionin pool tested, HTH or PTH (data not shown). Two different strains of each of these three sensitive species, and of *E. amylovora* as a negative control, showed comparable results. A radial diffusion assay showed that the fractions 34 to 60, which were positive for the presence of protein but negative for HTHs, did not contain antibacterial activity when tested with up to 125 μg of protein per well against *X. c.* pv. *vesicatoria* (data not shown).

To quantitate the antibacterial activity of PTH and HTH, bacteria were grown in broth in the presence of different concentrations of thionins and growth was measured turbidimetrically and by CFU counting. All bacteria in the control wells grew to densities of approximately 10^9 to 10^{10} CFU/ml within the period of testing. As expected, only the two strains of *C. m.* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus* and *X. c.* pv. *vesicatoria* were sensitive, whereas none of the other species showed growth inhibition (Table 1). Toxicity levels of PTH, HTH, HTH-1 and HTH-2 for the sensitive species appeared to be very similar, only minor differences could be detected (Table 2). These micro-plate assays indicated that thionins exhibit bactericidal rather than bacteriostatic activity for *C. m.* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus* and *X. c.* pv. *vesicatoria*, as the OD_{620} dropped below the OD at the start of the experiment and no colonies were formed after plating on solid medium. A nutrient broth (NB) was used instead of trypticase-soy-broth (TSB), to be able to compare the results of our toxicity assays with previously published data on the toxicity of PTHs [Fernandez de Caleya *et al.*, 1972]. For all the bacterial species tested, the results using either medium were closely similar (data not shown).

Table 1. Bacteria tested in this work in radial diffusion assays (RDA) and micro-plate broth dilution assays (BDA) and results from these experiments

Microorganism	Strain[a]	Host[b]	RDA[c]	BDA[d]
C. m. subsp. *michiganensis*	PD520 (= NCPPB1468)	*Lycopersicon esculentum*	+	+
	PD1386 (=NCPPB1064)	*L. esculentum*	+	+
C. m. subsp. *sepedonicus*	PD1381 (=IPO270)	*Solanum tuberosum*	+	+
	PD37 (=IPO498)	*S. tuberosum*	+	+
E. amylovora	PD269 (=IPO108)	*Craetagus* sp.	-	-
	PD1387 (=IPO295)	*Pyrus* sp.	-	-
E. c. subsp. *atroseptica*	PD230 (=IPO161)	*S. tuberosum*	-	-
	PD1385 (=IPO281)	*S. tuberosum*		-
E. c. subsp. *carotovora*	PD877 (=IPO857)	*S. tuberosum*		-
	PD878 (=IPO858)	*S. tuberosum*		-
E. chrysanthemi	PD226 (=IPO502)	*S. tuberosum*	-	-
	PD483 (=IPO871)	*S. tuberosum*		-
E. salicis	PD1382 (=Dsk147)	*Salix* sp.	-	-
	PD1383 (=Dsk152)	*Salix* sp.		-
P. solanacearum	PD134 (=IPO674)	*S. tuberosum*	-	-
	PD445 (=IPO933)	*S. tuberosum*		-
P. s. pv. *tabaci*	PD1616 (=NCPPB1408)	*Nicotiana tabacum*		-
	PD1617 (=NCPPB1427)	*N. tabacum*		-
P. s. pv. *tomato*	PD170 (=NCPPB1106)	*L. esculentum*		-
	PD828 (=IPO920)	*L. esculentum*	-	-
X. c. pv. *vesicatoria*	PD1389 (=No.81-18)	*Capsicum annuum*	+	+
	PD1390 (=No.XV35)	*L. esculentum*	+	+

[a] PD, collection Netherlands Plant Protection Service, Wageningen; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; IPO, collection DLO Research Institute for Plant Protection, Wageningen; Dsk, collection DLO Institute for Forrest tree and Nature Research, Wageningen and No., collection University of Florida, IFAS, Bradenton, Florida, USA

[b] host plant from which the culture was isolated

[c] RDA, radial diffusion assay; +, inhibition zone present and -, inhibition zone absent when tested with up to 125 μg PTH and HTH per well

[d] BDA, micro-plate broth dilution assay; +, growth inhibition and -, no growth inhibition, as

Table 2. Toxicity of thionins for sensitive plant pathogenic bacteria in μg/ml as determined by pour plating

Strain		PTH		HTH		HTH-1		HTH-2	
		MIC[a]	MBC[b]	MIC	MBC	MIC	MBC	MIC	MBC
C. michiganensis subsp. *michiganensis*	PD520	64	128	32	64	64	64	32	64
	PD1386	32	64	16	64	32	64	16	64
C. michiganensis subsp. *sepedonicus*	PD37	16	32	16	32	16	32	16	32
	PD1381	32	64	16	32	16	32	8	32
X. campestris pv. *vesicatoria*	PD1389	16	16	16	16	16	16	8	16
	PD1390	16	16	16	32	8	16	8	8

[a] MIC, minimal inhibitory concentration, the concentration at which growth was inhibited, as determined by CFU counting

[b] MBC, minimal bactericidal concentration, the concentration at which more than 99.9% of the inoculum is no longer able to form colonies.

2.5 Discussion

Our primary objective was to study the potential use of the type 1 HTHs for engineering bacterial disease resistance into crops because these thionins are the best characterized at the molecular level. Nucleotide and amino acid sequences of HTHs have been published before, and hence could serve as an ideal starting point in the isolation and construction of HTH encoding sequences. However, the toxicity of the HTHs from barley endosperm for plant pathogenic bacteria was not tested before. To this end, these were isolated from barley endosperm, together with the PTHs from wheat endosperm for which toxicity data were available. Because of the ease and quality of the purification, type 1 thionins were recovered from the lipoprotein complexes present in petroleum-ether extracts. This yielded an almost pure and biologically active thionin preparation in a few steps.

The type 1 HTHs were previously shown to be a mixture of two highly homologous proteins, α- and ß-HTH [Redman and Fisher, 1969]. HTH-1 and HTH-2 may represent α- and ß-HTH respectively, according to the elution from cation-exchange columns [Redman and Fisher, 1969]. No differences in mobility on SDSureaPAGE and acidPAGE could be detected for all the thionin pools examined, probably due to the high overall homology in molecular weight and charge [reviewed in García-Olmedo *et al.*, 1989] and the limited resolution of the gel system used.

The type 1 thionins from wheat endosperm were shown to be toxic for a number of bacteria [Fernandez de Caleya *et al.*, 1972; Stuart and Harris, 1942], some of which are pathogenic for plants. The possible mode of action is absorption onto the cell membrane, causing a change in permeability by the formation of ion-channel pores that result in leakage, as was shown for yeasts [Okada and Yoshizumi, 1973] and for a mouse fibroblast cell line [Oka *et al.*, 1992]. These studies were performed with the PTHs. As a fast assay to determine the toxicity of HTHs, a radial diffusion assay was used. Our results demonstrated that by this method only three bacterial species of the nine tested were sensitive for the HTHs and PTHs. The sensitive bacteria were: *C. m.* subsp. *michiganensis*, the causal agent of bacterial canker on tomato, *C. m.* subsp. *sepedonicus*, the causal agent of ring rot on potato and *X. c.* pv. *vesicatoria*, the causal agent of a spot disease on tomato and pepper. The same results were obtained by testing in broth in micro-plates.

Apart from *C. m.* subsp. *michiganensis* (MIC 450 μg/ml, MBC 450 μg/ml), *C. m.* subsp. *sepedonicus* (MIC 1 μg/ml, MBC 1 μg/ml) and an undefined pathovar of *X. campestris* (MIC 56 μg/ml, MBC 110 μg/ml), also *E. amylovora* (MIC 540 μg/ml, MBC 540 μg/ml) and *P. solanacearum* (MIC 5 μg/ml, MBC 5 μg/ml) were reported to be sensitive at the indicated doses [Fernandez de Caleya *et al.*, 1972]. Our results with PTH, HTH, HTH-1 and HTH-2 showed a tenfold higher toxicity for *C. m.* subsp. *michiganensis*, but slightly lower toxicity for *C. m.* subsp. *sepedonicus* and surprisingly no toxicity for *P. solanacearum*. Toxicity of thionins for *E. amylovora* was not tested at the high doses indicated above. The reported difference of *P. solanacearum* in sensitivity for PTHs is in agreement with the results of others, who also did not find toxicity of PTHs against *P. solanacearum* when tested with up to 500 μg/ml PTH (Broekaert, personal communication). Differences might have been caused by differences in strain and race specificity. The strains we used, PD134 and PD445, were race 3 isolates, whereas the strain and race specificity used by Fernandez de Caleya *et al.* [1972] was not indicated. A direct comparison is further hampered by a number of experimental differences. The toxicity assays reported earlier were performed in a nutrient broth with crude PTHs isolated from an unknown cultivar of *T. aestivum*. Differences in toxicity between thionins isolated from different cultivars might occur and could explain the observed discrepancies. However, radial diffusion assays performed with crude PTHs isolated from another, unrelated wheat cultivar, *T. aestivum* cv. Kraka, showed similar results (data

not shown). In addition, the PTHs isolated from the wheat cultivars Manitou [Jones and Mak, 1976] and Manitoba No. 3 [Ohtani *et al.*, 1975] were identical in amino acid sequence. Differences in toxicity resulting from the use of different growth media can be excluded because no significant differences, resulting from the use of TSB or NB, were observed.

Fernandez de Caleya *et al.* [1972] also investigated the difference in toxicity of α-PTH (a mixture of α1- and α2-PTH) and ß-PTH, purified by ion-exchange chromatography according to Fisher *et al.* [1968], and found species-dependent differences for *P. solanacearum* and *X. c.* pv. *phaseoli*. We could not demonstrate such species-dependent differences in toxicity of the two HTH fractions for the sensitive bacterial species.

In conclusion, our results show that HTHs from barley and PTHs from wheat endosperm show comparable toxicity for *C. m.* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus* and *X. c.* pv. *vesicatoria*, the causal agents of bacterial diseases in solanaceous crops. Minimal inhibitory concentrations (MIC) ranged from 16 to 64 μg/ml and the minimal bactericidal concentrations (MBC) were usually twice the MICs. Hence, both the PTHs [Fernandez de Caleya *et al.*, 1972] and the HTHs (this study) are potent antibacterial proteins only needed in relatively low concentrations to kill plant pathogenic bacteria. The genes encoding the HTHs have good prospects for genetic engineering bacterial disease resistance in Solanaceae, which lack these proteins.

We have made several gene constructs encoding different HTH precursor proteins to study the feasibility of engineering bacterial disease resistance into solanaceous crops. These have been introduced in tobacco, tomato and potato and the expression and biological activity of the HTHs produced in these transgenic plants will be discussed in the following chapters.

Acknowledgements

D.F. was supported by grants from the Dutch Programmatic Business orientated Technology Stimulation fund (PBTS) and from the Dutch Programme Committee Agricultural Biotechnology (PcLB). We are indebted to Gerard Bredemeijer and Henk Burg for helpful suggestions on chromatography of proteins and to Ab van Kammen and Lous van Vloten-Doting for helpful discussion. Toxicity assays were performed with permission of the Netherlands Plant Protection Service, P.O.Box 9102, 6700 HC Wageningen, The Netherlands.

2.6 References

Anzai H, Yoneyama K, Yamaguchi I: Transgenic tobacco resistant to a bacterial disease by the detoxification of a pathogenic toxin. Mol Gen Genet 219: 492-494 (1989).

Balls AK, Hale WS, Harris TH: A crystalline protein obtained from a lipoprotein of wheat flour. Cereal Chem 19: 279-288 (1942).

Békés F: A study of purothionin isolated from the petroleum ether extract of wheat flour. Acta Aliment 6: 39-57 (1975).

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 defence mechanism of plants. EMBO J 7: 1559-1565 (1988).

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).

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).

De la Fuente-Martínez JM, Mosqueda-Cano G, Alvarez-Morales A, Herrera-Estrella L: Expression of a bacterial phaseolotoxin-resistant ornithyl transcarbamylase in transgenic tobacco confers resistance to

Pseudomonas syringae pv. *phaseolicola*. Bio/Technology 10: 905-909 (1992).

Ebrahim-Nesbat F, Behnke S, Kleinhofs A, Apel K: Cultivar-related differences in the distribution of cell-wall-bound thionins in compatible and incompatible interactions between barley and powdery mildew. Planta 179: 203-210 (1989).

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).

Fisher N, Redman DG, Elton GAH: Fractionation and characterization of purothionin. Cereal Chem 45: 48-57 (1968).

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, Vuyck DH (eds), Agricultural biotechnology in focus in the Netherlands, pp. 39-46. Pudoc, Wageningen (1990).

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 of Plant Mol & Cell Biol 6: 31-60 (1989).

Jones BL, Mak AS: Amino acid sequences of the two α-purothionins of hexaploid wheat. Cereal Chem 54: 511-523 (1976).

McFarland J: The nephelometer: an instrument for estimating the numbers of bacteria in suspensions used for calculating the opsonic index and for vaccines. J Amer Med Assoc 49: 1176-1178 (1907).

Ohtani S, Okada T, Kagamiyama H, Yoshizumi H: The amino acid sequence of purothionin A, a lethal toxic protein for brewer's yeasts from wheat. Agric Biol Chem 39: 2269-2270 (1975).

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).

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).

Redman DG, Elton GAH: Reduction and re-oxidation of the purothionins. J Sci Food Agric 20: 546-549 (1969).

Redman DG, Fisher N: Purothionin analogues from barley flour. J Sci Food Agric 20: 427-432 (1969).

Sawyer RL: Potatoes for the developing world. International Potato Center, Lima, Peru (1984).

Stuart LS, Harris TH: Bactericidal and fungicidal properties of a crystalline protein isolated from unbleached wheat flour. Cereal Chem 19: 288-300 (1942).

Swank RT, Munkres KD: Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal Biochem 39: 462-477 (1971).

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).

CHAPTER 3

Design and construction of synthetic hordothionin genes

(with F. Heidekamp and L. Van Vloten-Doting)

3.1 Abstract

Hordothionins (HTHs) are small antimicrobial proteins from barley endosperm previously shown to be toxic *in vitro* for a number of bacteria pathogenic on tomato and potato (chapter 2). These HTHs are processed in barley from larger precursor proteins consisting of three distinct domains, an amino-terminal signalpeptide, followed by the mature HTH and a carboxy-terminal acidic peptide. HTH gene constructs, encoding different precursor proteins, were obtained by ligation of a number of synthetic DNA fragments. These were derived after conversion of oligonucleotides, having 10 to 12 complementary nucleotides at the 3'-ends, into double stranded DNA by DNA polymerase. This enzymatical method was optimized to render full-length DNA fragments of reliable nucleotide sequence. The codon usage and the 5'-ends of the genes were altered for optimal translation in solanaceous crops. With respect to the different pathogenic bacteria aimed at, constructs were designed to study expression of the antibacterial HTH in the cytosol and the intercellular spaces (apoplast) of leaves or vascular tissue. To reveal their potential in engineering bacterial disease resistance into crops that lack these proteins, all constructs were designed for convenient cloning in plant expression vectors.

3.2 Introduction

The type 1 α- and ß-hordothionins (HTHs) from the endosperm of barley (*Hordeum vulgare* L.) are small ($M_r \approx 5000$), highly basic proteins that exhibit, among others, antibacterial activity against a number of plant pathogens *in vitro* [Fernandez de Caleya *et al.*, 1972; Florack *et al.*, 1990; chapter 2]. In barley these thionins are encoded as precursor proteins consisting of three distinct domains: an amino-terminal signalpeptide (SP) of 24 amino acids, involved in transition of the precursor HTH protein into the endoplasmic reticulum (ER), followed by the mature highly basic HTH of 45 amino acids exhibiting the antibacterial activity and a carboxy-terminal acidic peptide (AP) of 64 amino acids with unknown function [Hernández-Lucas *et al.*, 1986; Ponz *et al.*, 1986; Rodriguez-Palenzuela *et al.*, 1988]. HTHs have been shown to be toxic for *Clavibacter michiganensis* subspecies (subsp.) *michiganensis*, a bacterial pathogen invading the vascular tissue of tomato causing bacterial canker, *C. m.* subsp. *sepedonicus*, the causal agent of ring rot in potato affecting primarily the tubers and *Xanthomonas campestris* pathovar (pv.) *vesicatoria*, the causal agent of a spot disease in tomato and pepper affecting leaves, fruits and the vascular tissue (chapter 2). The introduction and expression of (precursor) HTH encoding sequences in solanaceous crops was chosen as a model system to study the feasibility of engineering resistance against these plant pathogenic bacteria.

The use of synthetic genes for research purposes is growing fast [Gröger *et al.*, 1988]. Automated synthesis of nucleic acids has advanced tremendously in the past ten years and genes can now easily be obtained by chemical synthesis, provided that they are moderate in size and the amino acid sequences of the encoded proteins are known. Chemical synthesis of a gene has a number of advantages when compared to cDNA or genomic cloning, e.g.:

1. Codons can be selected to optimize the expression of the gene in a heterologous host.
2. Codons can be chosen in such a way as to place unique restriction sites at intervals throughout the gene to enable future exchange of fragments (modular mutagenesis).
3. Unique restriction sites can be created in the flanking, untranslated region to facilitate successive cloning steps.
4. Provisions for the placement of the synthetic gene with respect to promoter, operator, ribosome binding site and transcription terminator can all be included in the design.

Because of these advantages, fully synthetic HTH gene constructs encoding the mature α- and ß-HTH protein only (Type A), and constructs encoding the mature HTH fused to a SP (Type B), were designed for optimal expression in solanaceous crops [Florack *et al.*, 1990]. Type C HTH gene constructs which encoded the full-length precursor protein, were partly derived from Type A and B constructs (see also chapter 4).

A number of methods have been described to construct synthetic genes from chemically synthesized oligonucleotides. The most simple method is to synthesize oligonucleotides corresponding to much or all of both strands of the desired gene, and subsequently anneal and ligate them into a suitable vector (for examples see Bell *et al.*, 1988; Ferretti *et al.*, 1986; Wosnick *et al.*, 1987). Another method uses two oligonucleotides with short overlap at the 3'-ends, followed by an enzymatical conversion into double stranded DNA [Rink *et al.*, 1984; Rossi *et al.*, 1982]. This enzymatic approach reduces the number of oligonucleotides to be synthesized by as much as 40%. A disadvantage is that the efficiency of obtaining full-length double stranded DNA fragments is only 25 to 40% [Rossi *et al.*, 1982], whilst the correctness of the nucleotide sequence obtained after conversion has to be checked.

We here describe a detailed outline of the design of Type A and B HTH gene constructs, which were build by stepwise ligation of DNA fragments obtained using an optimized enzymatical conversion of two overlapping synthetic oligonucleotides, and Type C constructs which were derived from these.

3.3 Materials and methods

3.3.1 Design of Type A and B hordothionin gene constructs

Type A and B constructs were chemically synthesized. The Type A constructs pAα and pAß, encoding the mature α- and ß-HTH protein preceded by an ATG start codon, were designed based on the published sequences of the two type 1 barley endosperm thionin cDNA clones, pTH1 and pTH2 respectively [Hernández-Lucas *et al.*, 1986; Ponz *et al.*, 1986]. Type B constructs, pBα and pBß, encode a precursor protein containing the mature α- and ß-HTH fused to the SP of a the type 2 barley leaf thionin, derived from cDNA pDB4 [Bohlmann and Apel, 1987]. The translation initiation regions in these four synthetic genes were optimized according to Lütcke *et al.* [1987]. In addition, the codon usage of the α- and ß-HTH cDNA clones pTH1 and pTH2 was altered, either to create unique restriction sites throughout the coding region, or in favor of use in solanaceous crops. A codon usage table of tomato and potato was compilated of 11 sequences available from the EMBL Nucleotide Sequence Data Library (release 1988). Unique *Eco*RI and *Hpa*I restriction sites were created at the 5'-ends, and unique *Bam*HI and *Pst*I sites at the 3'-ends of the constructs to facilitate successive cloning steps. Nucleotide and amino acid sequences derived after optimization were analyzed with the PC/GENE program (GENOFIT Software, Geneva, Switzerland).

3.3.2 Oligonucleotide synthesis and purification

Oligonucleotides were synthesized on a BioSearch Cyclone DNA synthesizer using solid-phase ß-phosphoramidite chemistry [Beaucage and Caruthers, 1981] and phosphorylated using T_4 polynucleotide kinase and a mixture of ATP and [γ-^{32}P]ATP [Maniatis *et al.*, 1982]. Oligonucleotides exceeding 22 nucleotides in length were purified by preparative 10% polyacrylamide gelelectrophoresis (PAGE) in the presence of 7 M urea [Maniatis *et al.*, 1982]. After separation DNA was visualized by autoradiography and ethidium-bromide

staining and correct-length oligonucleotides were excised and eluted from the gel by overnight incubation at 37°C in a buffer containing 500 mM NH_4CH_3COO, 10 mM $Mg(CH_3COO)_2$, 1 mM EDTA and 0.1% SDS and further purified by spun-column chromatography on Sephadex G-50 [Maniatis *et al.*, 1982]. The amount of oligonucleotide was determined spectrophotometrically ($1A_{260}$ = 30 μg/ml).

3.3.3 Second strand synthesis

Equimolar amounts of the phosphorylated oligonucleotides with 10 to 12 overlapping nucleotides at the 3'-ends (25 pmol each), were mixed, heated at 100°C for 3 min and quickly cooled on ice for 5 min. The solution was made 10 mM Tris-HCl (pH 7.9), 6.6 mM $MgCl_2$, 6.6 mM ß-mercapthoethanol, 60 mM NaCl and 0.25 mM for all four deoxynucleotidetriphosphates in a total volume of 30 μl. After adding one unit of the large fragment of *Escherichia coli* DNA polymerase (Klenow, New England Nuclear), the reaction mix was incubated for 30 min at 37°C and heat inactivated by incubation for 3 min at 90°C. Double stranded DNA fragments were fractionated by 8% PAGE and visualized by autoradiography and ethidium-bromide staining of the gel. pBR322 digested with *Sau*3A1 and filled-in with α^{32}P-dCTP, dATP, dGTP and dTTP by incubation with Klenow identical as described above, was run on the same gel as a DNA size marker. Full-length fragments, as deduced from comparison with the DNA size marker, were excised from the gel, electroeluted in dialysis tubing in a buffer containing 20 mM Tris-CH_3COOH (pH 7.8), 10 mM $NaCH_3COO$ and 1 mM EDTA and further purified by spun-column chromatography. Fragments were ligated in Bluescribe pSK+-vector (Stratagene Inc., La Jolla, USA), transformed into *E. coli* XL1-Blue (recA-(*rec*A1, *lac*-, *end*A1, *gyr*A96, *thi*, *hsd*R17, supE44, *rel*A1, {F'proAB, *lac*I^Q, *lac*Z△M15, Tn10}), Stratagene Inc., La Jolla, USA) and verified by DNA sequence analysis using the dideoxy-mediated chain termination method [Sanger *et al.*, 1977], Sequenase (United States Biochemicals) and ^{32}P-dATP (Amersham).

3.3.4 Assembly of Type A and B hordothionin gene constructs

pAα, pAß, pBα and pBß DNA fragments were divided into a number of subfragments, each bordered by unique restriction sites. Oligonucleotides were synthesized for part of the upper and lower strand of these fragments. After conversion into double stranded DNA fragments, cloning and verification of the nucleotide sequences, those composing the different constructs were ligated stepwise to ultimately render the genes.

3.3.5 Design and assembly of Type C hordothionin gene constructs

Type C constructs were derived from the type 1 HTH cDNA clone pC263 (kindly provided by D. Brandt, Carlsberg Research Center, Copenhagen, Denmark), which was isolated from a cDNA library made from RNA of barley (cv. Bomi) endosperm. This cDNA clone lacked the SP encoding sequence and is nearly identical in nucleotide sequence to the *Hth-1* gene described previously [Rodriguez-Palenzuela *et al.*, 1988]. The Type C constructs, pC1α and pC1ß, were made by ligation of the *Sau*96I/*Bam*HI fragment of pC263 to the *Eco*RI/*Dde*I fragments of pBα and pBß respectively, using a synthetic adapter composed of (5'-TAAGTTG-3') and (5'-GGCCAAC-3'). pC2α was obtained by ligation of the *Eco*RI/*Pst*I fragment of pBß to the *Pst*I/*Bam*HI fragment of pC263.

3.4 Results

3.4.1 Design of Type A and B hordothionin gene constructs

For optimal expression in solanaceous crops the codon usage of Type A and B HTH gene constructs was optimized. To this end the codon usage of a number of genes originating from tomato and potato were compilated (Table 1, column COMP). The codons present in the HTH mRNA's pTH1 and pTH2 that were underrepresented in these crops were altered in favor of codons present in potato and tomato mRNA's. For example, the codon AAC encoding an asparagine residue which is present several times in pTH1, is underrepresented in *Solanaceae* compared to the other possible asparagine codon AAT (AAC : AAT = 10.4 : 35.2; Table 1) and therefore this codon is changed into the favorable AAT.

Table 1. Codon usage table. Frequencies (per one thousand) of codon usages are summed up for STPATG1, STPI2G, LEWIPI, LEWIPII, STLS1G, STPIIIR2, STRNA01, LEBIOBR, STPIIIR1, LEPG2AR and LEETHYBR (EMBL Nucleotide Sequence Data Library, release 1988) and are listed under **COMP** (based on a total of 6239 amino acids). The codon usage table published recently by Wada *et al.* [1990] is listed under **PUBL** (based on 54 genes from potato and tomato with a total of 13717 amino acids).

Amino acid	Codon	COMP	PUBL	Amino acid	Codon	COMP	PUBL
Ala	GCT	13.1	41.4		CTC	11.4	10.8
	GCC	5.2	14		CTA	16.2	6.4
	GCA	18.0	25.2		CTG	6.9	3.3
	GCG	2.4	3.3	Lys	AAA	50.7	28.3
Arg	CGT	2.3	9.5		AAG	27.8	32.7
	CGC	1.7	2.6	MET	ATG	32.9	27.4
	CGA	2.8	3.9	Phe	TTT	34.8	27.3
	CGG	1.8	0.7		TTC	16.7	17.5
	AGA	14.5	10.4	Pro	CCT	8.6	17
	AGG	14.1	8.8		CCC	8.6	8.8
Asn	AAT	35.2	31.2		CCA	15.3	24.6
	AAC	10.4	17.8		CCG	2.6	2
Asp	GAT	18.7	29.8	Ser	TCT	16.6	17.4
	GAC	10.5	15.7		TCC	8.6	10.4
Cys	TGT	20.7	11.4		TCA	16	17.8
	TGC	11.6	11.6		TCG	3.9	3.2
Gln	CAA	30.4	23.5		AGT	13.9	12.8
	CAG	8.6	9.0		AGC	7.0	11.7
Glu	GAA	24.7	33.5	Thr	ACT	17.0	26
	CAG	14.7	24.6		ACC	8.3	10
Gly	GGT	9.0	24.8		ACA	17.5	17.7
	GGC	10	12.8		ACG	2.3	2.6
	GGA	14.9	35.7	Trp	TGG	13.9	14.8
	GGG	8	7.2	Tyr	TAT	33.4	15.9
His	CAT	15.8	10.4		TAC	11.4	21.3
	CAC	11	5.6	Val	GTT	20.6	33.3
Ile	ATT	26.1	30.0		GTC	5.3	9.6
	ATC	15.8	14.7		GTA	17.6	10.3
	ATA	26.7	9.1		GTG	19.4	16.2
Leu	TTA	31.5	12.8	---	TAA	26.9	2.5
	TTG	32.5	23.6		TGA	17.2	1.2
	CTT	19.9	24.3		TAG	8.1	0.3

As an example all the changes made in the design of the Type B HTH gene construct pBα are illustrated in Table 2 and printed in italics. The obtained fragments were divided into a number of subfragments flanked by unique restriction sites and for each of these, two oligonucleotides were developed which were converted into a double stranded DNA fragment.

Table 2. Codon usage for part of the type 1 thionin cDNA clone pTH1 [Ponz *et al.*, 1986] encoding the SP and mature α-HTH, and for the corresponding part of the synthetic Type B HTH gene construct pBα which was optimized for expression in solanaceous crops. Frequencies of codons for these two are listed and differences due to codon usage adaptation in the synthetic gene are in italics. The first Val codon GGC in pTH1 and the Ile codon AGT in pBα are not counted.

Amino acid	Codon	pTH1	pBα	Amino acid	Codon	pTH1	pBα
Ala	GCT	1	1		*CTC*	*2*	*1*
	GCC	0	0		CTA	1	1
	GCA	1	1		*CTG*	*1*	*0*
	GCG	0	0	*Lys*	*AAA*	*3*	*1*
Arg	CGT	1	1		*AAG*	*2*	*4*
	CGC	1	1	MET	ATG	1	1
	CGA	0	0	Phe	TTT	0	0
	CGG	0	0		TTC	1	1
	AGA	1	1	*Pro*	*CCT*	*1*	*2*
	AGG	2	2		*CCC*	*1*	*0*
Asn	*AAT*	*0*	*2*		CCA	0	0
	AAC	*2*	*0*		CCG	0	0
Asp	GAT	0	0	*Ser*	TCT	0	0
	GAC	0	0		TCC	0	0
Cys	TGT	3	3		TCA	0	0
	TGC	6	6		TCG	0	0
Gln	*CAA*	*1*	*3*		*AGT*	*2*	*3*
	CAG	*2*	*0*		*AGC*	*2*	*1*
Glu	GAA	2	2	*Thr*	*ACT*	*0*	*1*
	CAG	0	0		*ACC*	*1*	*0*
Gly	GGT	1	1		ACA	2	2
	GGC	*3*	*0*		ACG	0	0
	GGA	*2*	*6*	Trp	TGG	0	0
	GGG	*1*	*0*	Tyr	TAT	0	0
His	CAT	0	0		TAC	1	1
	CAC	0	0	*Val*	*GTT*	*1*	*2*
Ile	ATT	0	0		*GTC*	*2*	*1*
	ATC	*0*	*1*		GTA	1	1
	ATA	*1*	*0*		GTG	2	2
Leu	TTA	2	2	---	TAA	0	0
	TTG	1	1		TGA	0	0
	CTT	*2*	*4*		TAG	-	-

3.4.2 Construction of Type A, B and C hordothionin genes

The oligonucleotides used in this work for the construction of the synthetic DNA fragments composing the different Type A and B HTH gene constructs, have been listed in Table 3. They were designed with unique restriction endonuclease recognition sites at the 5'-ends

(Table 3, underlined) and short complementary nucleotides at the 3'-ends for annealing and priming second strand synthesis. Differences in nucleotide sequence of the oligonucleotides due to the differences in amino acid sequence of α- and ß-HTH, are indicated in boldface.

Table 3. Oligonucleotides synthesized for the construction of the four Type A and B HTH gene constructs. The oligonucleotides referred to in the first column (oli) and those with complementary nucleotides at the 3'-ends listed in the last column (compl), were pairwise converted into double stranded DNA fragments. The unique restriction sites used in cloning experiments are given above the recognition sites (underlined). Differences in nucleotide sequence between C and I, and G and J due to differences in amino acid sequence between the α- and ß-HTH are in boldface.

oli[1]	length[2]	nucleotide sequence and restriction sites (underlined)	compl[3]
		EcoRI HpaI	
A	30	5'-ctggaattcgttaacaatggtgtgtttact-3'	B
		AvaII	
B	30	5'-aggaccaatccaaggataagtaaacacacc-3'	A
		AvaII	
C	32	5'-tggtccttgaacaagtgcaagtagaaggaaag-3'	D
		MaeI	
D	35	5'-cctagagtactcctgcagcaactctttccttctac-3'	C,I
		MaeI	
E	32	5'-tctaggaagaaattgctacaatctttgccgcg-3'	F
		MstI	
F	36	5'-tgcgccataacttttgagcaccacgaacgcggcaaag-3'	E
		MstI	
G	68	5'-gttatgcgcaaatgcttgtaggtgtaaactcacaagtggactaaagtgcccttcaagtttccctaagt-3'	H
		PstI BamHI	
H	24	5'-ctgcagggatccctacttagggaa-3'	G,J
		AvaII	
I	32	5'-tggtccttgaacatgtgcaagtagaaggaaag-3'	D
		MstI	
J	68	5'-gttatgcgcaggagtttgtaggtgtaaactcacaagtagcggaaagtgccctacaggattccctaagt-3'	G
		EcoRI HpaI	
K	37	5'-ggaattcgttaacaatggcacccagcaagagtattaa-3'	L
		StyI	
L	43	5'-accaaggatgagaacacaaatgaccacactcttaatactcttg-3'	K
		EcoRI HpaI	
M	21	5'-ggaattcgttaacaatgaaga-3'	N
		PstI	
N	22	5'-cctgcagcaactcttcattgtt-3'	M

[1], oligonucleotide identifier
[2], length in nucleotides
[3], identifier for oligonucleotide with complementary 3'-ends

Preparative gelelectrophoresis resulted in purified full-length oligonucleotides (Figure 1A). Different annealing conditions, enzymes, concentrations, reaction buffers and conditions for second strand synthesis were evaluated with respect to the yield of full-length double stranded DNA fragments (data not shown). This resulted in the procedure as described in the Materials and methods section. For small fragments, up to 95% of the input single stranded oligonucleotides were converted into double stranded DNA, while larger oligonucleotides were converted into full-length fragments with an efficiency of 50%. As an example the conversion of the oligonucleotides A and B (Table 3) into fragment AB, and oligonucleotides

K and L into KL, is shown in Figure 1B. Comparison with the DNA size marker (Figure 1B, lane M) revealed that A and B were converted into a 50 basepair fragment and K and L into a 68 basepair fragment, which were the sizes expected upon correct conversion (see also Table 3). The reliability of the method was checked by DNA sequence analysis of a large number of independent recombinants derived after cloning of one of the fragments. Only one mismatch was found among the 1500 nucleotides sequenced.

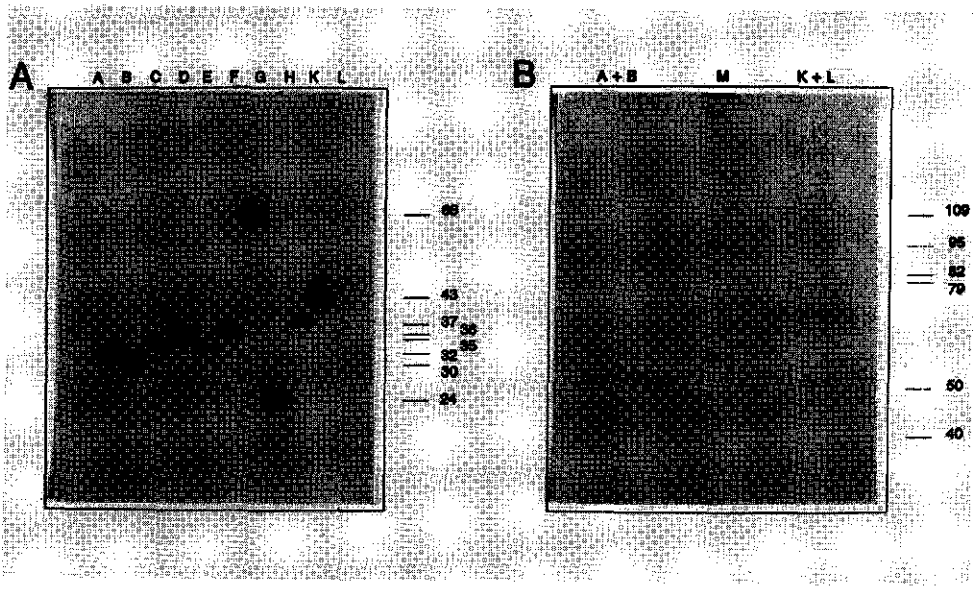


Figure 1. Construction of synthetic HTH gene constructs. A. Autoradiograph of [γ-^{32}P]ATP-labelled oligonucleotides purified by preparative gelelectrophoresis and used in the construction of the Type B HTH gene construct pBα, after separation by 10% PAGE in the presence of urea. Lane A, oligo A (30-mer); lane B, oligo B (30-mer); lane C, oligo C (32-mer); lane D, oligo D (35-mer); lane E, oligo E (32-mer); lane F, oligo F (35-mer); lane G, oligo G (68-mer); lane H, oligo H (24-mer), lane K, oligo K (37-mer) and lane L, oligo L (43-mer). Sizes in nucleotides are indicated at the right. B. Autoradiograph of second strand synthesis reaction mixtures after separation by 12% PAGE. Lane A+B, second strand synthesis of oligonucleotides A and B, having 12 complementary nucleotides at the 3'-ends, into a double stranded fragment (AB) of 50 basepairs; lane M, DNA size marker: pBR322 digested with *Sau*3A1 and filled-in with ^{32}P-dCTP and dATP, dTTP and dGTP by Klenow; lane K+L, second strand synthesis of oligonucleotides K and L, having 12 complementary nucleotides at the 3'-ends, into a double stranded fragment (KL) of 68 basepairs. Sizes in basepairs are indicated at the right.

C

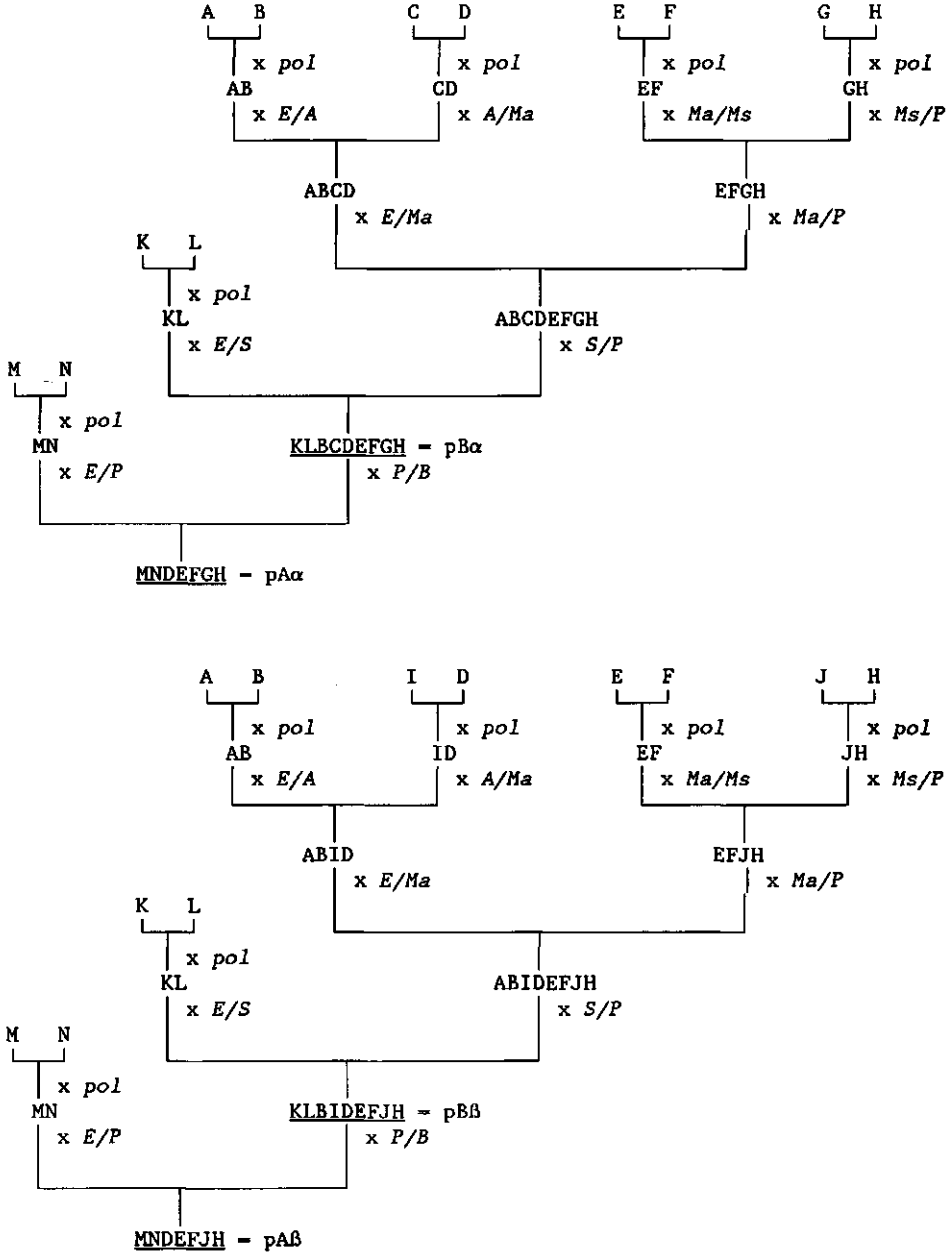


Figure 1. continued. C. Flow charts of the constructions of the synthetic Type A and B HTH gene constructs pAα, pAß, pBα and pBß. The different oligonucleotides involved in the synthesis of double stranded DNA fragments are indicated by the letter identifiers listed in Table 3. All the fragments that were ligated to render the different gene constructs are represented by the two letters of the oligonucleotides involved in the second strand synthesis reaction (see Table 3). Abbreviations: A, *Aval*I; B, *Bam*HI; H, *Hpa*I; Ma, *Mae*I; Ms, *Mst*I; P, *Pst*I; pol, DNA polymerase; S, *Sty*I. The completed HTH gene constructs are underlined.

The Type B HTH gene constructs were made by stepwise ligation of five DNA fragments, each obtained after enzymatical conversion of two oligonucleotides (see Table 3, first and last column) into double stranded DNA. The Type A HTH gene constructs were subsequently made from these Type B constructs by ligation of a chemically synthesized DNA fragment at the *Pst*I site. The oligonucleotides involved and strategy followed in the construction of each of these genes are outlined in Figure 1C.

The nucleotide and amino acid sequences of the different Type A and B HTH gene constructs, derived after ligation of the fragments with optimized nucleotide sequences (outlined in Figure 1C) are depicted in Figure 2. All the nucleotides changed in the synthetic genes can be deduced by comparison of the nucleotide sequences with that of the type 1 HTH gene *Hth-1* [Rodriguez-Palenzuela *et al.*, 1988] shown in the upper line of the figure. All the changes made do not affect the amino acid sequences (compare nucleotide sequences in Figure 2A with deduced amino acid sequences in 2B).

The Type C1 HTH gene constructs were obtained by ligation of the AP encoding fragment of the cDNA clone pC263 at the artificial *Dde*I site preceding the TAG stop codon in the Type B HTH gene constructs using an *Dde*I/*Sau*96I adapter with compatible ends. This strategy resulted in the same nucleotide sequence at the junction of the mature HTH and AP as originally present in the cDNA (see Figure 2A and 2B). The Type C2 HTH gene construct was derived from pC263 by ligation of the SP encoding sequence of pBα at the *Pst*I site. The general structures of a thionin cDNA and the different Type A, B and C HTH gene constructs described here is depicted in Figure 2C.

In summary, for ease in cloning several unique restriction sites were created along the coding sequence and in the untranslated 5'- and 3'-regions of the constructs. With respect to cloning in *E. coli* vector Bluescribe pSK+ and the binary *A. tumefaciens* plant expression vector pCPO5 (see chapter 4), unique *Eco*RI and *Hpa*I sites were created at the 5'- and unique *Bam*HI and *Pst*I sites at the 3'-untranslated regions of all genes.

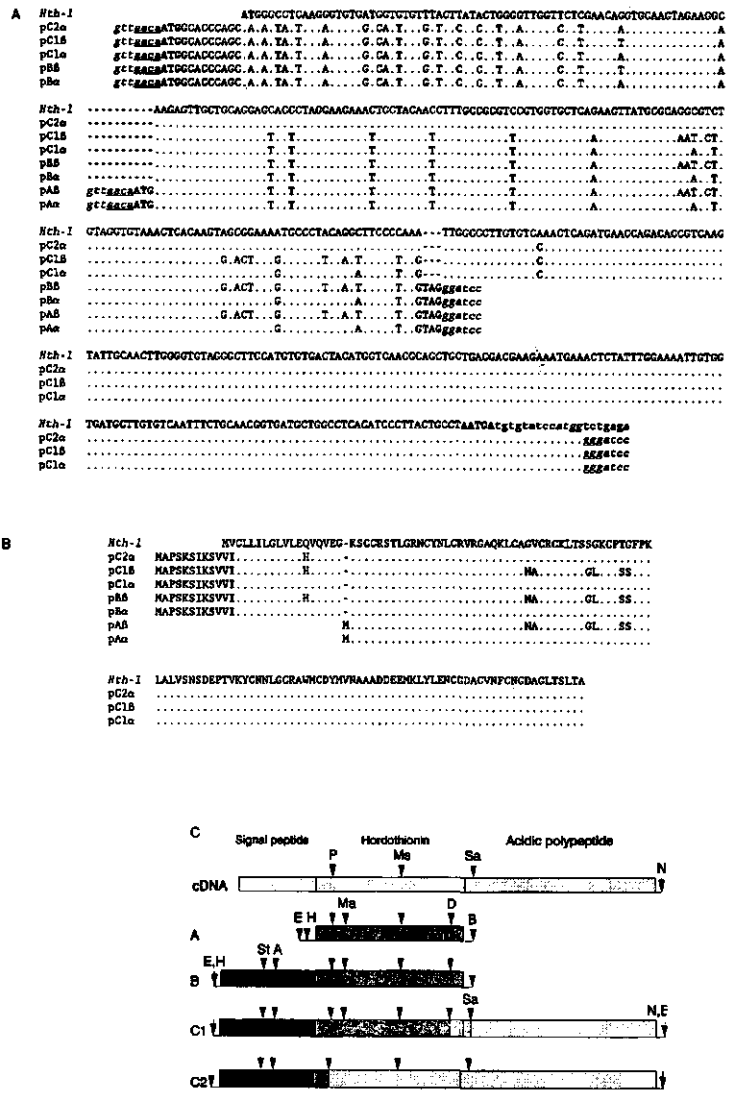


Figure 2. Structures and sequences of the different HTH gene constructs made. A and B. Nucleotide and amino acid sequences of pAα, pAß, pBα, pBß, pC1α, pC1ß and pC2α. For comparison, the corresponding sequence of the α-hordothionin gene from barley, *Hth-1* [Rodriguez-Palenzuela *et al.*, 1988], is shown on top. Translation start and stopcodons are printed in boldface. Flanking *Bam*HI, *Hpa*I and *Nco*I restriction sites used for cloning, are in italics and the optimized translation initiation region [Lütcke *et al.*, 1987] is underlined. Sequences in common with the *Hth-1* gene are indicated by dots (.). C. Schematic drawing of the composition of a type 1 HTH cDNA [Hernández-Lucas *et al.*, 1986; Ponz *et al.*, 1986] and Type A, B and C HTH gene constructs. 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 HTH gene 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.

3.5 Discussion

The antibacterial type 1 thionins from barley endosperm, which were previously shown to be toxic for three bacteria pathogenic on tomato and potato [Florack *et al.*, 1990; chapter 2], were chosen as a model system to investigate the feasibility of engineering bacterial disease resistance into solanaceous crops by the introduction and expression of antibacterial genes. Three types of constructs, encoding different precursor HTHs, were of interest to study such an approach. Chemical synthesis of the Type A and B HTH gene constructs was favored because:

1. At the start of this research program, the nucleotide sequences of three type 1 HTH cDNA clones [Hernández-Lucas *et al.*, 1986], and the amino acid sequence of the mature α-HTH [Ozaki *et al.*, 1980] were known thus making the design and chemical synthesis of HTH encoding sequences facile. cDNA or genomic libraries of barley were not available to us. The time necessary to get these coding sequences by a cloning approach, followed by successive adaptations by site-directed mutagenesis needed to make the different Type A and B HTH gene constructs, together with their moderate size, voted in favor of chemical synthesis of the genes. The very potent polymerase chain reaction (PCR) technique was just discovered at the start of this research program and hence not yet considered for isolating or constructing these genes.
2. We wanted to use gene constructs encoding the highly homologous α- and ß-HTH, which only differ by 7 amino acids in the SP and mature protein domain (see Figure 2 B), because a previous study [Fernandez de Caleya *et al.*, 1972] suggested differences in toxicity of the α- and ß-HTH towards bacteria. Taking advantage of the unique restriction sites created along the coding sequence, several fragments made by chemical synthesis could be used for the construction of both α- and ß-HTH gene constructs, minimizing the cost of oligonucleotide synthesis. Only those fragments differing in amino acid composition were made separately (see flowchart Figure 1C).
3. Computer analysis of the primary and secondary structures of the HTH precursors, as deduced from the three available cDNA sequences, indicated that the SP domain did not fulfill all the criteria of a SP sequence [Kikuchi and Ikehara, 1991]: an amino-terminal region with a net positive charge; a core region with at least nine hydrophobic residues to span the membrane; a helix breaking or a large polar residue situated four to eight residues before the proteolytic cleavage site and a cleavage consensus site -A-X-B ↓ Y-, where A is an uncharged residue (Val; see Figure 2 B), X is any residue (Glu), B is an uncharged residue with small side chains (Gly), ↓ is the signal peptidase cleavage site and Y is the first residue of the mature protein (Lys). The SP of all type 1 HTHs lacked a net positively charged amino-terminus which enhances SP function [Vlasuk *et al.*, 1983; von Heijne, 1984]. For this reason the mature α- and ß-HTH encoding sequences were supplemented with the SP encoding sequence of a type 2 leaf thionin cDNA (pDB4) previously isolated from a cDNA library made from RNA of barley leaves [Bohlmann and Apel, 1987]. The SP sequence of this type 2 thionin does have a stretch of positively charged amino acids at the amino-terminus and a large homology with the SP sequences of type 1 HTHs, resulting in a similar putative signalpeptidase cleavage site (see Figure 2B).
4. The type 1 HTH genes originate from barley which is a monocotyledon, and were only expressed in the endosperm [see review by García-Olmedo *et al.*, 1992 and references therein]. Our aim was to express these genes in different organs of tomato and potato, which are dicotyledonous species belonging to the family of the *Solanaceae*. In order to obtain a high level of expression in these crops, the codon usage of the genes encoding α- and ß-HTH, was adapted. Codons in pTH1 and pTH2 that were scarcely used in

solanaceous crops were altered. The codon usage table applied was based on plant DNA sequences known in the beginning of 1988 (Table 1, COMP). Recently a more comprehensive codon usage compilation was made by Wada *et al.* [1990] which turned out not significantly to differ from the one we used years before (Table 1, PUBL).

The designed nucleotide sequences were analyzed by computer for the presence of stretches of more than five identical nucleotides, that could lead to problems in sequence analysis, potential hairpin structures, inverted repeats and palindromic sequences. If present these sequences were altered.

Recent studies in yeast and *E. coli* indicated that the codon bias is probably related to the expression levels of the genes involved [Kurland, 1991]. Genes with low codon usage bias are likely to be weakly expressed. In addition, the codon context may also have an effect on translation [Bulmer, 1990; Ernst, 1988; Valle *et al.*, 1992]. Moreover, the translatability of a plant mRNA can also be strongly influenced by the stability of the messenger and hence the secondary structure [Vancanneyt *et al.*, 1990]. These last aspects were not considered in our design of early 1988.

For application in genetic engineering it is essential that the proteins are expressed in the biologically active form and in reasonable amounts in those plant tissues and compartments where the bacteria are present. Our ultimate goal is to achieve resistance in tomato against *C. m.* subsp. *michiganensis* and *X. c.* pv. *vesicatoria* and in potato against *C. m.* subsp. *sepedonicus*. *C. m.* subsp. *michiganensis*, the causal agent of bacterial canker, infects tomato plants via cuttings or wounds and is initially restricted to the xylem vessels. The intercellular spaces of adjacent xylem parenchyma and phloem elements are invaded at advanced stages of pathogenesis [Wallis, 1977]. *X. c.* pv. *vesicatoria*, the causal agent of a spot disease, infects tomato, and pepper plants, via stomata or wounds on leaves. Subsequent spread is restricted to the intercellular spaces [Stall and Cook, 1966]. *C. m.* subsp. *sepedonicus*, the causal agent of ring rot, infects potato plants primarily through tuber wounds, but also through wounds in stems, roots, stolons, or other plant parts. Subsequently it invades vessels, xylem parenchyma and adjacent tissue [Stefani, 1989]. HTH gene constructs were designed for expression intracellularly and extracellularly, respectively aimed at bacteria causing rot and bacteria spreading through the intercellular spaces or invading the vascular tissue. The Type A HTH gene constructs in which both the SP and AP sequences were omitted (see Figure 2C), were designed to study the expression of the HTH in the cytosol. Type B and C HTH gene constructs containing a SP were designed to obtain extracellular targeting. All synthetic genes were placed under the control of the strong CaMV 35S promoter, which is active in almost all plant tissues [Benfey and Chua, 1990]. Expression of these HTH gene constructs in tobacco and their subcellular localization are described in chapter 4. Experiments to determine the potential of these HTH genes for engineering resistance into tomato against *C. m.* subsp. *michiganensis* and *X. c.* pv. *vesicatoria* are described in chapter 5.

Acknowledgements

D.F. was supported by a grant from the Dutch Program committee Agricultural Biotechnology (PcLB). We want to thank Dirk Bosch and Ab van Kammen for critical reading of the manuscript and helpful discussion and Anders Brandt for the gift of pC263.

3.6 References

Beaucage SL, Caruthers MH: Deoxynucleoside phosphoramidites- a new class of key intermediates for

deoxypolynucleotide synthesis. Tetrahedron Lett 22: 1859-1862 (1981).
Bell LD, Smith JC, Berbyshire R, Finlay M, Johnson I, Gilbert R, Slocombe P, Cook E, Richards H, Clissold P, Meredith D, Powell-Jones CH, Dawson KM, Carter BL, McCullagh KG: Chemical synthesis, cloning and expression in mammalian cells of a gene coding for human tissue-type plasminogen activator. Gene 63: 155-163 (1988).
Benfey PN, Chua N-H: The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. Science 250: 959-966 (1990).
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).
Bulmer M: The effect of context on synonymous codon usage in genes with low codon usage bias. Nucl Acids Res 18: 2869-2873 (1990).
Ernst JF: Codon usage and gene expression. Tibtech 6: 196-199 (1988).
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).
Ferretti L, Karnik SS, Khorana HG, Nassal M, Oprian DD: Total synthesis of a gene for bovine rhodopsin. Proc Natl Acad Sci USA 83: 599-603 (1986).
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 biotechnology in focus in the Netherlands, pp. 39-46. Pudoc, Wageningen (1990).
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), Genes involved in plant defense, pp. 283-302. Springer-Verlag, Wien (1992).
Gröger G, Ramalho-Ortigao F, Seliger H: A comprehensive list of chemically synthesized genes. Nucl Acids Res 16: 7763-7771 (1988).
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).
Kikuchi M, Ikehara M: Conformational features of signal sequences and folding of secretory proteins in yeasts. Tibtech 9: 208-211 (1991).
Kurland CG: Codon bias and gene expression. FEBS Lett 285: 165-169 (1991).
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).
Maniatis T, Fritsch EF, Sambrook J: Molecular cloning, a laboratory manual. Cold Spring Harbor (1982).
Ozaki Y, Wada K, Hase T, Matsubara H, Nakanishi T, Yoshizumi H: Amino acid sequence of a purothionin homologue from barley flour. J Biochem 87: 549-555 (1980).
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).
Rink H, Liersch M, Sieber P, Meyer F: A large fragment approach to DNA synthesis: total synthesis of a gene for the protease inhibitor eglin c from the leech *Hirudo medicinalis* and its expression in *E. coli*. Nucl Acids Res 12: 6369-6387 (1984).
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).
Rossi JJ, Kierzek R, Huang T, Walker PA, Itakura K: An alternate method for synthesis of double-stranded DNA segments. J Biol Chem 257: 9226-9229 (1982).
Sanger F, Nicklen S, Coulson AR: DNA sequencing with the chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467 (1977).
Stall RE, Cook AA: Multiplication of *Xanthomonas vesicatoria* and lesion development in resistant and susceptible pepper. Phytopathol 56: 1152-1154 (1966).
Stefani E: *In situ* detection of *Clavibacter michiganensis* ssp. *sepedonicus* in potato stems using the fluorescent antibody technique. Phytopathol medit 28: 53-56 (1989).
Valle RPC, Drugeon G, Devignes-Morch M-D, Legocki AB, Haenni A-L: Codon context effect in virus translational readthrough. A study in vitro of the determinants of TMV and Mo-MuLV amber suppression. FEBS Lett 306: 133-139 (1992).
Vancanneyt G, Rosahl S, Willmitzer L: Translatability of a plant-mRNA strongly influences its accumulation in transgenic plants. Nucl Acids Res 18: 2917-2921 (1990).
Vlasuk GP, Inouye S, Ito H, Itakura K, Inouye M: Effects of the complete removal of basic amino acid residues from the signal peptide on secretion of lipoprotein in *Escherichia coli*. J Biol Chem 258: 7141-7148 (1983).

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).

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).

Wallis FM: Ultrastructural histopathology of tomato plants infected with *Corynebacterium michiganense*. Physiol Plant Pathol 11: 333-342 (1977).

Wosnick MA, Barnett RW, Vincentini AM, Erfle H, Elliott R, Sumner-Smith M, Mantei N, Davies RW: Rapid construction of large synthetic genes: total chemical synthesis of two different versions of the bovine prochymosin gene. Gene 60: 115-127 (1987).

CHAPTER 4

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

Plant Molecular Biology 24: 83-96 (1994)

(with W.G. Dirkse, B. Visser, F. Heidekamp and W.J. Stiekema)

4.1 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- (SP) and pro- (AP) sequences 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 chimeric 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, although 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* subspecies (subsp.) *michiganensis* appeared similar to that of the HTH purified from barley endosperm.

4.2 Introduction

Thionins are a family of low-molecular-weight ($M_r \approx 5000$) proteins that have been identified in various plant species (recently reviewed in [García-Olmedo *et al.*, 1989,1992]). They can be divided into at least five different types, based on the number of amino acids, the net charge and the number of disulphide bonds present in the mature protein [Castagnaro *et al.*, 1992]. 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 ß-PTH and the HTHs of two proteins, designated α- and ß-HTH (reviewed in [García-Olmedo *et al.*, 1989]). 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 [Hernández-Lucas *et al.*, 1986; Ponz *et al.*, 1983,1986]. The type 1 PTHs and HTHs have been shown to exhibit anti-microbial properties against a number of bacteria, fungi and yeasts [Bohlmann *et al.*, 1988; Fernandez de Caleya *et al.*, 1972; Florack *et al.*, 1990,1993; Hernández-Lucas *et al.*, 1974; Okada and Yoshizumi, 1970,1973; Stuart and Harris, 1942].

The type 1 PTHs have also been shown to be toxic *in vitro* for a number of plant pathogenic bacteria at relatively low concentrations [Fernandez de Caleya *et al.*, 1972]. Toxicity differed slightly depending on the PTH used, α- (a mixture of α1- and α2-PTH) or

ß-PTH for two of the bacteria tested [Fernandez de Caleya *et al.*, 1972]. 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 [Florack *et al.*, 1993]. 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 [Florack *et al.*, 1990; García-Olmedo *et al.*, 1989]. 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* pathovar (pv.) *tabaci* 153 and *P. s.* pv. *syringae* [Carmona *et al.*, 1993]. 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 [Carmona *et al.*, 1993].

In this chapter we report the introduction of seven synthetic and semi-synthetic gene constructs encoding different type 1 α- and ß-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.

4.3 Materials and methods

4.3.1 Hordothionin plasmid constructions

The seven HTH gene constructs used in this work are represented in Figure 1. Type A and B constructs were made chemically. The Type A constructs pAα and pAß were designed based on the published sequences of two type 1 HTH cDNA clones, pTH1 and pTH2 encoding α- and ß-HTH respectively [Hernández-Lucas *et al.*, 1986]. The Type B constructs pBα and pBß were derived from the Type A constructs by the addition of the SP encoding sequence of a type 2 leaf thionin cDNA clone, pDB4 [Bohlmann and Apel, 1987]. 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 [Wada *et al.*, 1990]. Some of the codons were also altered to create unique restriction sites at interval throughout the gene (Figure 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.* [1987], and a *BamH*I site at the 3'-end, directly following the stopcodon (Figure 1A & B). All synthetic genes were made by ligation of fragments derived after enzymatical conversion of 3'-end overlapping oligonucleotides as described previously [Florack *et al.*, 1990]. The Type C1 constructs pC1α and pC1ß, 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 pBß, 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 pBß to pC263, digested with *Pst*I and *Bam*HI. All gene constructs were initially cloned in Bluescribe pSK+ (Stratagene Inc., La Jolla, USA) and verified by sequencing using the dideoxy-mediated chain-termination method [Sanger *et al.*, 1977] 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 (Figure 2). This binary vector was derived from pPCV708 [Koncz *et al.*, 1990] 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 [Kay *et al.*, 1987], and the nopaline synthase (NOS) terminator sequence. The recombinant binary vectors were conjugated into *Agrobacterium tumefaciens* GV3101 (pMP90) or GV3101 (pMP90RK) [Koncz and Schell, 1986] by parental mating [van Larebeke *et al.*, 1974] using *Escherichia coli* HB101(pRK2013) [Ditta *et al.*, 1980] as helper strain when appropriate.

4.3.2 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.* [1985]. Shoots, regenerated in the presence of 100 mg/l kanamycin were rooted in MS-medium [Murashige and Skoog, 1962], 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.

4.3.3 Hordothionin expression and processing analysis

Total RNA was isolated from young leaves (approximately 5 cm) of greenhouse-grown plants essentially as described [De Vries *et al.*, 1991]. 10 μg of total RNA from each plant was separated on a 2% agarose gel in the presence of 6% (vol/vol) formaldehyde, transferred to membranes (GeneScreenPlus, New England Nuclear) for Northern analysis [Sambrook *et al.*, 1989] 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. Approximately 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 trichloroacetic 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 [Bradford, 1976].

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 [Towbin *et al.*, 1979] was performed using a polyclonal antiserum raised in a rabbit against HTHs, coupled to bovine serum albumin (BSA) to increase antigenicity [Deen *et al.*, 1990]. 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 analyzed twice in independent experiments.

Proteins were analyzed by SDS-tricine polyacrylamide gelelectrophoresis (SDS-tricine-PAGE) according to Schägger and Jagow [1987] and by acid PAGE as described previously

[Florack *et al.*, 1993]. Rainbow coloured low molecular weight protein markers were from Amersham. Separated proteins were transferred to polyvinylidine difluoride (Immobilon-P, Millipore) or nitrocellulose (BA 85, Schleicher & Schuell) membranes in a Trans-Blot Cell (Bio-Rad) and were analyzed by enhanced chemiluminescence Western blotting (ECL, Amersham), using the HTH antiserum and horse radish peroxidase-labelled secondary antibody, according to the manufacturer.

4.3.4 Subcellular localization analysis

Intercellular washing fluids 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 [de Wit and Spikman, 1982]. 100 μl intercellular fluid of each plant was analyzed by protein gel blotting as described above. Further analysis of the subcellular localization of HTH was established by plant cell fractionation of the same, aseptically grown, plants essentially as described [Denecke *et al.*, 1990]. After incubation for 20 h in culture medium, protoplasts (2 x 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 sec at 4°C) in 200 μl of the above mentioned buffer. 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 hr at 40°C and proteins were precipitated, resuspended in 100 μl 1% CH_3COOH and analyzed (25 μl of each fraction) by protein gel blotting for the presence of HTH as described above.

4.3.5 Analysis of biological activity

Protein extracts from approximately 200 g of leaf material from the selected plants described above were enriched for the HTH by selective water-ethanol treatment [Fisher *et al.*, 1968]. 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 blot immuno-assay. These extracts were tested for toxicity towards *Clavibacter michiganensis* subspecies (subsp.) *michiganensis* PD1386 (collection Dutch Plant Protection Service, Wageningen) in the micro-plate broth dilution assay as described previously [Florack *et al.*, 1993]. 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 x 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 tenfold dilutions in trypticase soy agar (Becton-Dickinson) in 24-well Cluster^{24} (Mark II, Costar) tissue culture plates.

4.4 Results

4.4.1 Hordothionin gene constructs and transformation

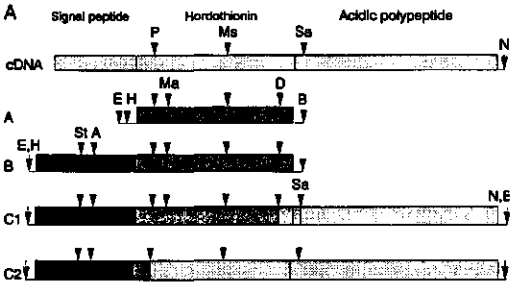
Gene constructs were prepared encoding different α- and ß-HTH precursor proteins to investigate the necessity of a SP, the AP or both for expression, extracellular targeting and biological activity. Constructs designated Type A code for the mature protein domain only, Type B for the mature protein with a SP and Type C for the full-length precursor protein (Figure 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 ß-HTH were preceded by the SP sequence derived from a type 2 barley leaf thionin [Bohlmann and Apel, 1987]. 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 [von Heijne, 1984]. Most of the SP sequence and the cleavage site remain unchanged, compared to the original SP sequence, due to the high homology (Figure 1B & C). In all these synthetic genes the regions surrounding the ATG-codon were optimized for translation initiation according to Lütcke *et al.* [1987]. 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 pBß construct to the *Pst*I site of the cDNA (Figure 1A). The nucleotide and amino acid sequences of all the constructs made are shown in Figure 1B and C.

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 (Figure 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.

4.4.2 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 analyzed expressed the gene. The level of HTH-mRNA varied depending on the type of construct used in the transformation event (Figure 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.



B.

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

Hth-1     ----------AAGAGTTGGTGCAGGAGCAGCCTAGGAAGAAACTGCTACAACGTTTGCCGCGTCCGTGGTGGTCAGAAGTTATGCGCAGGCGTCT
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       gtcgacgATG.....T..T..........T.......T..........T..........A.............AAT.CT.
pAα       gtcgacgATG.....T..T..........T.......T..........T..........A.............A..T.

Hth-1     GTAGGTGTAAACTCACAAGTAGCCGAAAATGCCCTACAGGCTTCCCCAAA---TTGGCCCTTGTGTCAAACTCAGATGAAGCAGACACCGTCAAG
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     TATTGCAACTTGGGGTGTAGGGCTTCCATGTGTGACTACATGGTCAACGCAGCTGCTGACGACGAAGAAATGAAACTCTATTTGCAAAATTGTGC
pC2α      ....................................................................................................
pC1ß      ....................................................................................................
pC1α      ....................................................................................................

Hth-1     TGATGCTTGTGTCAATTTCTGCAACGGTCATGCTGGCCTCAGATGCCTTACTGCCTAATGAtgtgtatccatggtctgaga
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.....-.....................MA.......GL...SS...
pC1α      MAPSKSIKSVVI.....................-.....................................................
pBß       MAPSKSIKSVVI.........H.....-.....................MA.......GL...SS...
pBα       MAPSKSIKSVVI.....................-.....................................................
pAß                                   M.....................MA.......GL...SS...
pAα                                   M.....................................................

Hth-1     LALVSNSDEPTVKYCNNLGCRAVNCDYNVRAAADDEEMKLYLENCGDACVNFCNGDAGLTSLTA
pC2α      ...............................................................
pC1ß      ...............................................................
pC1α      ...............................................................
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Figure 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, *Msp*I; N, *Nco*I; P, *Pst*I; Sa, *Sau*96I; St, *Sty*I. B and C. Nucleotide and amino acid sequences of pAα, pAß, pBα, pBß, pC1α, pC1ß 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 [Lütcke *et al.*, 1987] is underlined. Sequences in common with the *Hth-1* gene are indicated by dots (.).

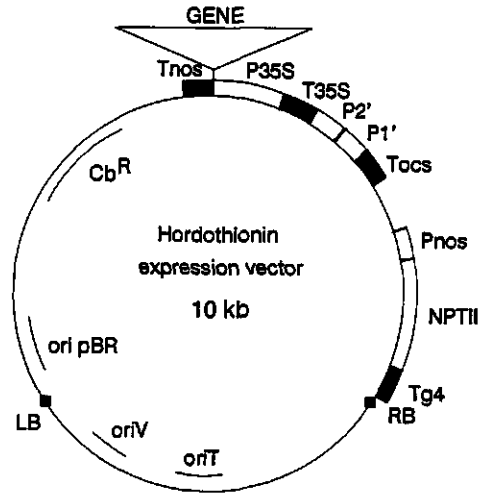


Figure 2. Representation of the binary vector pCPO5 containing the different HTH gene constructs. RB, right T-DNA border sequence; Tg4, T-DNA gene 4 terminator; NPTII, neomycin phosphotransferase II gene, selectable kanamycin resistance marker; Pnos, nopaline synthase promoter; Tocs, octopine synthase 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.

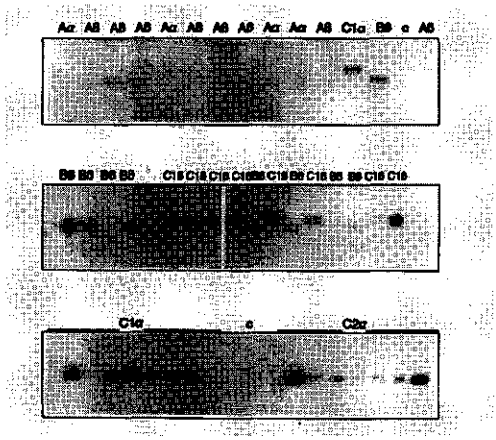


Figure 3. Autoradiographs of Northern blots containing total RNA of transgenic tobacco plants hybridized with HTH-specific probes. The construct type used for transformation is indicated on top of the lanes. The lane containing total RNA of a control kanamycin resistant plant is indicated by a "c".

HTH protein expression was studied of 48 plants with a Type A (pAα and pAß), 80 plants with a Type B (pBα and pBß) and 178 plants with a Type C (pC1α, pC1ß 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 [Okada and Yoshizumi, 1970]. 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 (approximately 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 analyzed 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 pBß construct had detectable HTH protein. In young tobacco leaves of plants with a Type B construct (pBα), HTH levels up to approximately 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 (Figure 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 Figure 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.

4.4.3 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 (Figure 5 A, lane Cα) showed a single band which comigrated with HTH-1 (Figure 5 A, lane HTH) purified from barley endosperm and which probably represents α-HTH [Florack *et al.*, 1993]. The apparent molecular weight was 5000 D, which is in agreement with that of the mature α-HTH encoded by these constructs as deduced from the amino acid sequence [Hernández-Lucas *et al.*, 1986; see Figure 1 C). The immunoblot of the same extract separated on an acid polyacrylamide gel (Figure 5 B, lane Cα) also showed one band comigrating with the biologically active HTH from barley endosperm (Figure 5 B, lane HTH). 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 (Figure 5 A & B, lane Bα). On the contrary, the immunoblots of protein extracts from ANF903401 (pAα; Figure 5 A and B, lane Aα) and from the control kanamycin resistant plant ANF900701 (Figure 5 A and B, lane Co) 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.

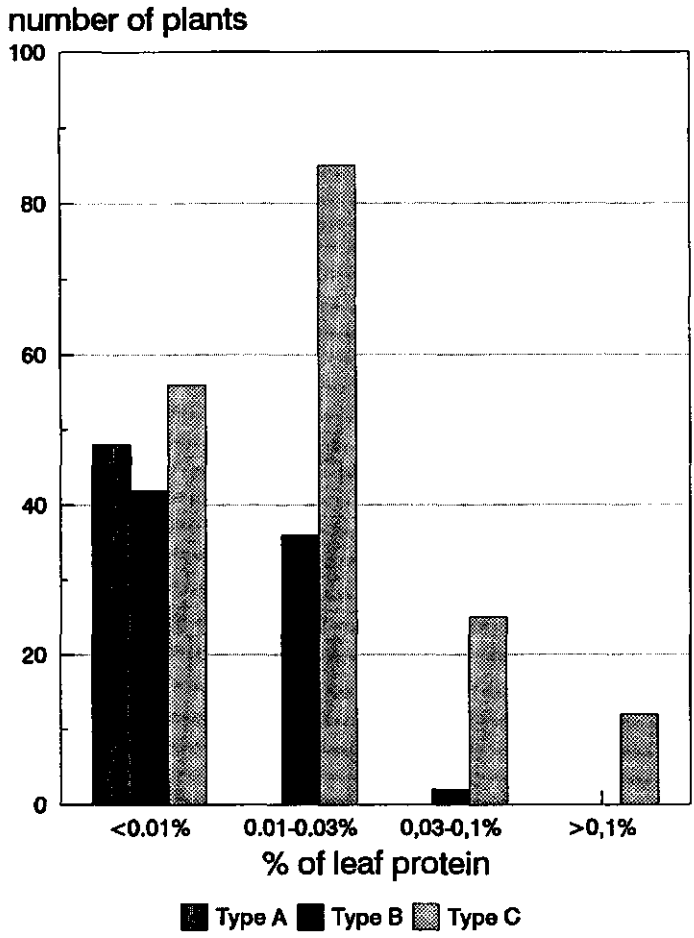


Figure 4. Distribution of transgenic tobacco plants according to the HTH protein expression level. Expression of 48 plants containing a Type A, 80 plants containing a Type B and 178 plants containing a Type C gene construct were analyzed by dot blot immuno-assays and the expression levels were divided 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.

4.4.4 Localization of HTH in transgenic tobacco

The same plants analyzed for HTH processing were used to study the subcellular localization of the mature HTH. Western blot analysis of intercellular washing fluids 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 (Figure 6 A & B, lanes Cα) indicated that HTH was present in the "cytosol", "microsome" and "membrane" fraction at an approximate ratio 1:5:5 and comigrated with the HTH from barley endosperm (Figure 6 A & B, lanes HTH). The apparent molecular weight was 5000 D, which is in agreement with the results from the processing analysis (Figure 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 (Figure 6 B, lanes Bα) and migrated slightly slower, confirming our previous findings. As expected, the immunoblots containing the fractions from the two control plants showed no bands (Figure 6 A & B, lanes Aα and Co).

The faint bands of higher molecular weight in the "microsome" and "membrane" fractions of ANF902932 (Figure 6 B, lanes Cα), 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.

4.4.5 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. m.* subsp. *michiganensis* in micro-plates. This bacterial plant pathogen was previously shown to be susceptible to HTHs [Florack *et al.*, 1993]. Serial twofold 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 (Figure 7). In contrast, extracts from plants containing the pAα construct were not toxic at similar protein levels.

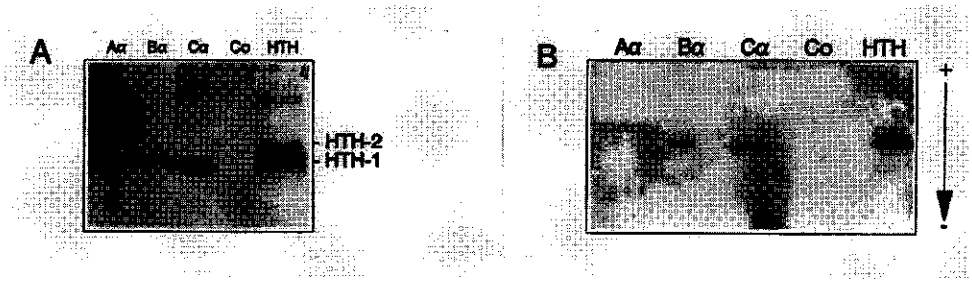


Figure 5. Immunoblots of leaf proteins from tobacco plants transformed with the pAα, pBα and pC1α constructs after incubation with the HTH antibody. Lane Aα, ANF903401; lane Bα, ANF901710; lane Cα, ANF902932; lane Co, ANF900701, kanamycin resistant control plant and lane HTH, purified HTH from barley endosperm. A. Proteins were separated by SDS-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.

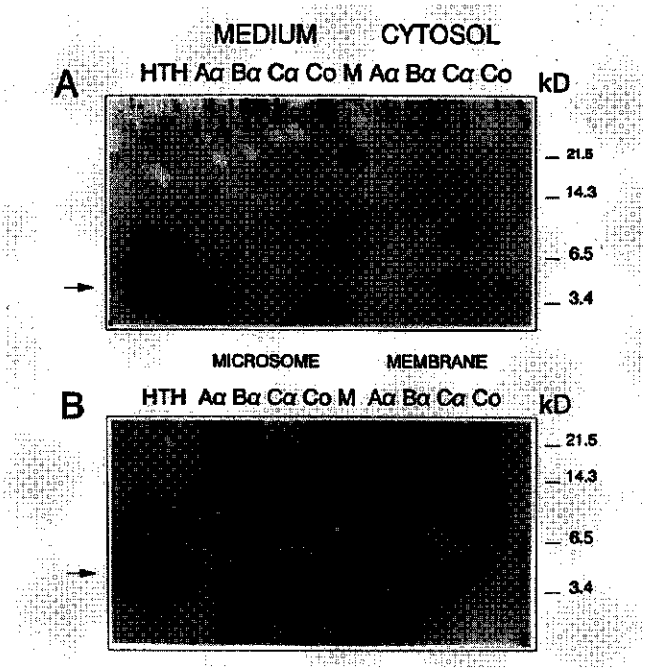


Figure 6. Immunoblots of the different fractions from tobacco protoplasts of plants transformed with the pAα, pBα and pC1α construct and control plants after incubation with the HTH antibody. All fractions were treated with dilute sulphuric acid. Lanes HTH, 100 and 50 ng HTH respectively; lanes Aα, ANF903401; lanes Bα, ANF901710; lanes Cα, ANF902932; lanes Co, ANF900701, control kanamycin resistant plant and lanes M, 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.

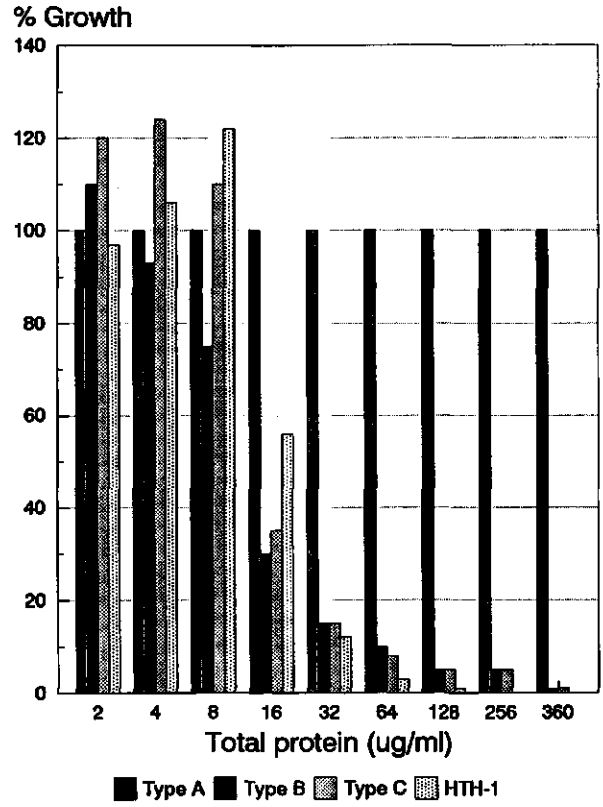


Figure 7. *In vitro* growth of the bacterial plant pathogen *Clavibacter michiganensis* subsp. *michiganensis* PD1386 in serial twofold dilutions of partially purified extracts from transgenic plants. Growth in extracts from plants containing the pBα construct (ANF901710) and the pC1α construct (ANF902932) was compared to growth in extracts from plants harboring 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 x 10^3 CFU/ml and plates were incubated for 24 h at 27°C before plating.

4.5 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 (Figure 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 [Chrispeels, 1991]) where conditions for disulphide bond formation are

favourable and enzymes like protein disulphide isomerase are present which catalyze the formation of these bonds [Freedman, 1989; Rothman, 1989]. 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 [Freedman, 1989] which in addition does not contain the enzyme protein disulphide isomerase [Rothman, 1989], 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 (Figure 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 pBß construct. Protein gel blotting revealed that the HTH had a molecular weight of approximately 5000 D, which indicates correct processing of both precursors (Figure 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 (Figure 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 [Woodget *et al.*, 1986]. This serine residue is flanked by a number of basic residues (see Figure 1 C), which enhance the kinetic parameters for protein kinase C phosphorylation [Woodget *et al.*, 1986]. 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 (Figure 5 A & B), 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 (Figure 6 A). 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 [Carbonero *et al.*, 1980; Ponz *et al.*, 1983] and immunogold-labelling performed on barley leaves which indicated that the (type 2) thionin was present in the vacuoles [Reimann-Philipp *et al.*, 1989] and cell wall [Bohlmann *et al.*, 1988]. These type 2 thionins were also not found in the intercellular spaces of barley leaves [Fisher *et al.*, 1989]. 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 (approximately 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 [Carrasco *et al.*, 1980; Oka *et al.*, 1992; Okada and Yoshizumi, 1973] cannot be excluded. The largest amount of HTH from the plant

with the pBα construct was in the "membrane" fraction (Figure 6 B, Bα membrane versus microsome) whereas in plants expressing the pC1α construct the ratio was equal for "microsome" and "membrane" fractions (Figure 6 B, Cα membrane and microsome), 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 chaperon 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.* [1978], who first used the term 'molecular chaperon' 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 (Figure 4, 5 & 6). Carmona *et al.* [1993] 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 harboring a genomic HTH gene construct under control of a CaMV 35S promoter [Carmona *et al.*, 1993], were similar to those found in our transgenic plants harboring 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. s.* pv. *tabaci* 153 and *P. s.* pv. *syringae* [Carmona *et al.*, 1993]. Recently, we demonstrated that the type 1 HTHs from barley are toxic for *C. m.* subsp. *michiganensis*, the causal agent of bacterial canker in tomato, *C. m.* 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 [Florack *et al.*, 1993], 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. s.* pv. *tabaci* NCPPB1408 (= PD1616) and *P. s.* pv. *tabaci* NCPPB1427 (= PD1617) were not sensitive for HTHs in contrast to *P. s.* pv. *tabaci* 153 used by Carmona *et al.* [1993]. This might be explained by differences in bacterial strains. Therefore 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. m.* subsp. *michiganensis*.

Experiments to determine the level of resistance of transgenic tomato plants, harboring a Type C HTH gene construct giving highest expression in tobacco, by infection with *C. m.* subsp. *michiganensis* and *X. c.* pv. *vesicatoria* and transgenic potato plants by infection with *C. m.* subsp. *sepedonicus* will be discussed in the next chapter.

Acknowledgements

D.F. was supported by grants from the Dutch Programme committee Agricultural Biotechnology (PcLB) and from the Dutch Programmatic Business orientated Technology Stimulation fund (PBTS). We are indebted to Dirk Bosch and Ab van Kammen for critical reading of the manuscript and helpful discussion, to Anders Brandt for the gift of pC263 and to Erik Dullaard, Dick Geurtsen, Johan Hulsman and Peter de Jong for taking care of transgenic plants. Assays with plant pathogenic bacteria were performed with permission of the Netherlands Plant Protection Service at Wageningen.

4.6 References

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).

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).

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).

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).

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. The Plant Journal 3: 457-462 (1993).

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).

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).

Chrispeels MJ: Sorting of protein in the secretory system. Annu Rev Plant Physiol Plant Mol Biol 42: 21-53 (1991).

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).

Denecke J, Botterman J, Deblaere R: Protein secretion in plant cells can occur via a default pathway. Plant Cell 2: 51-59 (1990).

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).

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 Pathol 21: 1-11 (1982).

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).

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).
Fisher N, Redman DG, Elton GAH: Fractionation and characterization of purothionin. Cereal Chem 45: 48-57 (1968).
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).
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 99: 259-268 (1993).
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 biotechnology in focus in the Netherlands, pp. 39-46. Pudoc, Wageningen (1990).
Freedman, RB: Protein disulphide isomerase: multiple roles in the modification of nascent secretory proteins. Cell 57: 1069-1072 (1989).
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), Genes involved in plant defense, pp. 283-302. Springer-Verlag, Wien (1992).
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 Surveys Plant Mol Cell Biol 6: 31-60 (1989).
Hernández-Lucas C, Fernandez de Caleya R, Carbonero P: Inhibition of brewer's yeasts by wheat purothionins. Appl Microbiol 28: 165-168 (1974).
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).
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).
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).
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).
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).
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).
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).
Murashige T, Skoog F: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497 (1962).
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).
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).
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).
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).
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).
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).
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).
Rothman JE: Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell 59: 591-601 (1989).
Sambrook J, Fritsch EF, Maniatis T: Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989).

Sanger F, Nicklen S, Coulson AR: DNA sequencing with the chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467 (1977).

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).

Stuart LS, Harris TH: Bactericidal and fungicidal properties of a crystalline protein isolated from unbleached wheat flour. Cereal Chem 19: 288-300 (1942).

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).

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).

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).

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).

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).

Supplementary material to chapter 4

A recent publication by Carmona *et al.* [1993] showed that transgenic tobacco plants containing an α-hordothionin gene with two introns, under the control of a CaMV 35S promoter and Nos terminator sequences, and exhibiting high hordothionin expression, had resistance to *Pseudomonas syringae* pv. *syringae* and *P. syringae* pv. *tabaci* 153. While the former is not pathogenic on tobacco under normal conditions, *P. syringae* pv. *tabaci* is the causal agent of a devastating disease called bacterial wildfire. Estimation of growth of this bacterium in leaves of transgenic and control plants upon infiltration of a bacterial suspension, indicated that growth was reduced at least tenfold in the transgenic plant exhibiting highest hordothionin expression when compared to bacterial growth in leaves of control plants [Carmona *et al.*, 1993]. Despite our finding that none of the *P. syringae* pv. *tabaci* strains we tested previously (PD1616=NCPPB1408 and PD1617=NCPPB1427) were sensitive to hordothionins *in vitro* [chapter 2], we decided to evaluate growth of this phytopathogen in our transgenic tobacco plants exhibiting high hordothionin expression. To this end five plants each of ANF900701 (control kanamycin resistant plant) and ANF900516 (HTH expression level approximately 4 μmol/kg) were infiltrated with a 1.6×10^4 CFU/ml bacterial suspension of *P. syringae* pv. *tabaci* PTBR2.024, a highly virulent strain causing wildfire on tobacco and grean beans [Engst and Shaw, 1992]. PTBR2.024 is rifampicillin resistant and was kindly provided by P. Shaw (University of Illinois-Champaign, Illinois, USA). The bacterial suspension was infiltrated using a syringe into the underside of fully-expanded leaves [Klement, 1963]. Infiltrated sectors were marked with a pencil and plants were kept at 28°C at 16 h daylength and 70% relative humidity. At daily intervals, three punches with a diameter of 0.5 cm were collected from infiltrated sectors and macerated in 200 μl peptone-phosphate buffer [McGuire *et al.*, 1986]. Serial tenfold dilutions were made and appropriate dilutions were plated in LB supplemented with 100 mg/l rifampicillin in microplates. After incubation of the plates at 28°C for two days, colonies were counted. The results are presented in Figure 8 and indicate that bacterial growth is not inhibited in either type of plants. This pathogen is restricted to the apoplast of leaves [Stewart, 1971]. The discrepancy between our results and those of Carmona *et al.* [1993] might have been the result of differences in bacterial strain, although inhibition of bacterial growth in the apoplast of leaves cannot be the result of a direct effect of the antibacterial hordothionin produced by the plant and the invading bacterium, since we clearly demonstrated that hordothionin is not secreted [chapter 4 and 5].

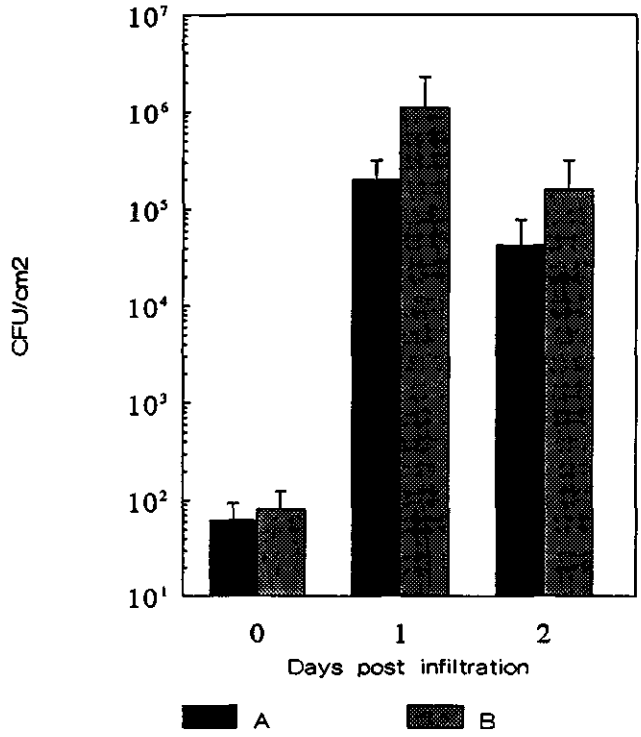


Figure 8. Growth of *Pseudomonas syringae* pv. *tabaci* PTBR2.024 in leaves of transgenic tobacco plants. A, ANF900701 (control kanamycin-resistant plant) and B, ANF900516 (transgenic plant exhibiting high hordothionin expression). Leaves were infiltrated with a 1.6 x 10^4 CFU/ml bacterial suspension and reisolated immediately following infiltration (day 0), and 1 and 2 days *post* infiltration. Appropriate serial tenfold dilutions were plated on rifampicillin-containing plates. Standard deviations are indicated by vertical bars.

References

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. The Plant Journal 3: 457-462 (1993).

Engst K, Shaw PD: Identification of a *lysA*-like gene required for tabtoxin biosynthesis and pathogenicity in *Pseudomonas syringae* pv. *tabaci* strain PTBR2.024. Mol Plant-Microbe Inter 5: 322-329 (1992).

Klement Z: Rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature 199: 299-300 (1963).

McGuire RG, Jones JB, Sasser M: Tween media for semiselective isolation of *Xanthomonas campestris* pv. *vesicatoria* from soil and plant material. Plant Disease 70: 887-891 (1986).

Stewart W: Isolation and proof of structure of wildfire toxin. Nature 229: 174-178 (1971).

CHAPTER 5

Expression of a full-length α-hordothionin gene in tomato

(with W.J. Stiekema and B. Visser)

5.1 Summary

A gene construct encoding an α-hordothionin precursor protein has been introduced in tomato (*Lycopersicon esculentum* cv. Moneymaker). The precursor protein, consisting of an amino-terminal signalpeptide, the mature α-hordothionin and a carboxy-terminal acidic peptide appeared to be correctly processed. The mature protein was expressed up to high levels in leaves and fruits of transgenic plants. No apparent effect on plant phenotype was observed and transgenic plants were self-fertile. Analysis of the intercellular washing fluids indicated that the mature protein was not secreted into the apoplast. Transgenic plants homozygous for the α-hordothionin gene were obtained after selfing of the primary transgenic plants and analysis of neomycin phosphotransferase II and α-hordothionin expression. The amount of the mature protein was slightly elevated in homozygous compared to hemizygous plants and expression of the gene was uniform among all plants of all three generations tested (T_1, T_2 and T_3). Homozygous plants exhibiting high α-hordothionin expression were tested for resistance against *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker on tomato, and *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of a foliage and fruit spot disease on tomato (and pepper). These two bacterial species were previously found to be susceptible to α-hordothionin *in vitro* (chapter 2). Transgenic plants had no enhanced resistance to *C. m.* subsp. *michiganensis*. In addition, no growth inhibition of *X. c.* pv. *vesicatoria* was observed in leaves of transgenic plants. However, when spray-inoculated with a bacterial suspension of the latter, transgenic tomato plants exhibiting high α-hordothionin expression showed only minor symptoms in contrast to control plants which were severely affected.

5.2 Introduction

Hordothionins (HTHs) are highly basic, low-molecular-weight ($M_r \approx 5000$) proteins from barley endosperm exhibiting antibacterial activity *in vitro* [Florack *et al.*, 1990; chapter 2]. Two HTHs are known, designated α- and ß-HTH, sharing high sequence homology. In barley, HTHs are synthesized as larger precursor proteins, consisting of a signalpeptide, the mature HTH and an acidic peptide [Hernández-Lucas *et al.*, 1986; Ponz *et al.*, 1986]. The signalpeptide, involved in transport of the precursor protein across the membrane of the endoplasmic reticulum (ER), is essential for mature protein expression in transgenic plants [chapter 4]. The acidic peptide is not essential but enhances accumulation of the mature protein at least tenfold and probably enables transport through membranous structures like the ER. In summary, expression of different α- and ß-HTH precursor proteins in tobacco indicated that only full-length gene constructs encoding all three domains, were highly expressed and that the precursor proteins encoded by these constructs were correctly processed into the mature α- or ß-HTH. *In vitro* growth inhibition experiments with partially purified α-HTH from leaves of transgenic tobacco plants, indicated that the protein was biologically active against *C. m.* subsp. *michiganensis* at the micromolar level [chapter 4].

In this chapter we describe the introduction and expression of an α-HTH precursor protein encoding sequence, pC2α [chapter 4], in tomato. In addition, we present the results of experiments performed to determine whether transgenic tomato plants exhibiting high α-HTH expression levels had acquired resistance to two pathogenic bacteria.

5.3 Materials and methods

5.3.1 Hordothionin gene construct, plant material and transformation

The recombinant plant expression vector pC2α [chapter 4], harboring a chimaeric semi-synthetic gene encoding a complete α-HTH precursor protein, was used for transformation of tomato. As a control, the empty plant expression vector pCPO5, conferring kanamycin resistance to plants, was used.

The Dutch true breeding tomato line Moneymaker (Rijk Zwaan, De Lier, the Netherlands), which is susceptible to *C. m.* subsp. *michiganensis* [Van den Bulk *et al.*, 1989] and *X. c.* pv. *vesicatoria* [unpublished results], was used in the transformation experiments. The tomato line Hawaii 7998, which is foliage-resistant to *X. c.* pv. *vesicatoria* [Jones and Scott, 1986; Scott and Jones, 1986] was kindly provided by Jeffrey Jones (University of Florida, Bradenton, USA).

Cotyledon explants of thirteen day-old aseptically grown seedlings of Moneymaker were transformed essentially as described [Koornneef *et al.*, 1986]. Shoots, regenerated in the presence of 150 mg/l kanamycin, were rooted on MS-medium [Murashige and Skoog, 1962], supplemented with 20 μg/l indole acetic acid, 200 mg/l cefotaxim, 200 mg/l vancomycin and 150 mg/l kanamycin. After rooting, plantlets were transplanted in normal potting compost, transferred to the greenhouse and grown to maturity.

5.3.2 Analysis of hordothionin expression

Transgenic tomato plants were evaluated for HTH expression in leaves by dot blot immunoassays and protein gel blotting as described [chapter 4]. Intercellular washing fluids were recovered from fully expanded leaves of selected transgenic tomato plants according to De Wit and Spikman [1982]. These were analyzed by dot blot immunoassays and protein gel blotting using a HTH antiserum. Expression of HTH in fruits was determined by cutting a fruit in half, placing it on a nitrocellulose membrane (BA85, Schleicher and Schuell) to allow protein binding, followed by a washing step and Western blot analysis [Towbin *et al.*, 1979].

5.3.3 Breeding for homozygosity

Transgenic plants were analyzed for the number of T-DNA loci by selfing primary transgenic plants (T_1) and analyzing the offspring of individual plants for segregation of the NPTII and HTH gene. Selection for kanamycin resistance was performed by spraying seedlings (third true leaf), in the greenhouse, with a 150 mg/l kanamycin solution three times on three subsequent days, as described [Weide *et al.*, 1989]. Seedlings were scored for the appearance of white sectors on treated leaves (kanamycin sensitive). Independently, all offspring plants were evaluated for HTH expression by the dot blot immunoassay. Kanamycin resistant (Kan^R) offspring plants (T_2) of primary transgenic plants that segregated 3:1 for Kan^R : Kan^S and HTH expression : no expression were selected for further breeding. A number of these Kan^R-T_2 plants were selfed, and the offspring of individual plants were analyzed as above. Plants that were homozygous for the NPTII and HTH gene were selected for the analysis of enhanced resistance.

5.3.4 Bacteria, plant inoculations and growth conditions

Analysis of resistance of transgenic tomato plants to *C. m.* subsp. *michiganensis* was performed in the greenhouse with transgenic and control plants essentially as described [Van Steekelenburg, 1985]. Inoculation was carried out in a randomized experiment with 9 replications. The bacterial strain used in this study was PD1386 (IPO 542, NCPPB1064) and was obtained from the Netherlands Plant Protection Service. This strain was previously found to be highly aggressive on Moneymaker [Van den Bulk *et al.*, 1989] and sensitive to α-HTH *in vitro* [chapter 2].

Growth of *X. c.* pv. *vesicatoria* in leaves of transgenic tomato plants and Hawaii 7998 plants, was determined by infiltration of a bacterial suspension, adjusted to 1 x 10^5 or 1 x 10^6 colony-forming units (CFU)/ml, into the underside of fully expanded leaves using a syringe as described [Jones and Scott, 1986]. Infiltration of plants was carried out in a growth chamber and plants were tested in a randomized experiment with 5 replications. The bacterial strain used in this experiment was PD1389 (IPO 922 = No. 81-18, an *Xcv*PT strain from the collection University of Florida) and was sensitive to α-HTH *in vitro* [chapter 2]. Infiltrated sectors were marked by pencil and treated plants were kept in a growth chamber at 29°C, 80% relative humidity and 12 h day / 12 h night. After 0, 2, 4, 7, 10 and 15 days, infiltrated leaflets were collected and three leaf discs with a diameter of 0.5 cm were macerated in peptone-phosphate buffer. Serial tenfold dilutions were plated on Tween A plates, a selective medium developed for the isolation of *X. c.* pv. *vesicatoria* from soil and plant material [McGuire *et al.*, 1986]. Plates were incubated at 28°C for three days and colonies with a fried-egg appearance (a white zone around a circular raised yellow colony), typical for *X. c.* pv. *vesicatoria*, were counted.

Analysis of foliage resistance of transgenic tomato plants was tested by spray-inoculation of plants with a bacterial suspension of *X. c.* pv. *vesicatoria*, adjusted to 1 x 10^8 CFU/ml, essentially as described [Scott and Jones, 1984]. The bacterial strain used in this experiment was the same as described above. Inoculation was carried out in a growth chamber and two plants each of a transgenic plant exhibiting high HTH expression and control kanamycin resistant plant were tested. Inoculated plants were kept at 29°C and > 90% relative humidity and 12 h day / 12 h night. After 13 days, plants were evaluated for the appearance of spots on leaves and removed.

5.4 Results

5.4.1 Hordothionin expression in tomato

Transformation of tomato (cv. Moneymaker) resulted in 12 independent transgenic plants harboring the pC2α gene construct and 5 independent plants harboring the pCPO5 gene construct. Dot blot immunoassays of leaf extracts indicated that almost all plants harboring pC2α expressed the gene, whereas control plants harboring pCPO5 showed, as expected, no HTH expression (data not shown). All plants were phenotypically normal and produced fruits after selfing. However, 2 out of the 12 pC2α-plants produced tiny fruits containing few seeds, probably caused by somaclonal variation or polyploidy.

Fruits of a number of these transgenic plants were analyzed by tissue blotting. Those from the transgenic plant which had the highest HTH expression in leaves, designated ALF900514-T_1, also appeared to exhibit highest HTH expression in fruits (Figure 1). The amount of HTH in leaves and fruits of individual transgenic plants was positively correlated (data not shown). Segregation analysis performed on 64 offspring plants (T_2) of ALF900514-

T_1, indicated that 46 were kanamycin resistant and 18 sensitive. This was confirmed by dot blot immunoassays and protein gel blotting of leaf extracts of these plants using the HTH antiserum. All plants but one, selected as kanamycin resistant, had high α-HTH contents (Figure 2), whereas kanamycin sensitive plants did not express the gene.

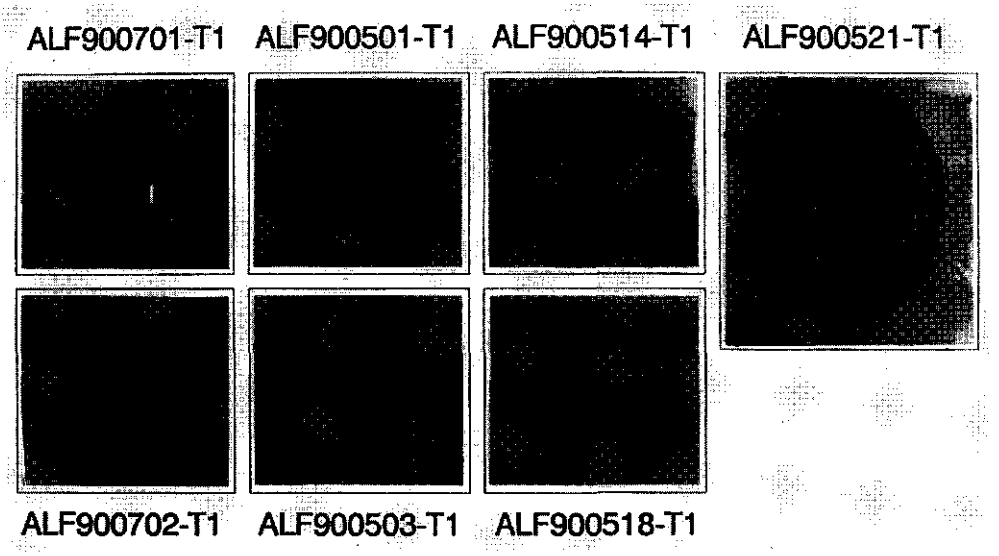


Figure 1. Tissue blots of ripe fruits of transgenic tomato plants. The blot was analysed using a hordothionin antiserum. ALF900701-T_1 and 900702-T_1 are control kanamycin resistant tomato plants harboring the pCPO5 gene construct; ALF 900501-T_1, 900503-T_1, 900514-T_1, 900518-T_1 and 900521-T_1 are transgenic tomato plants harboring the pC2α hordothionin gene construct.

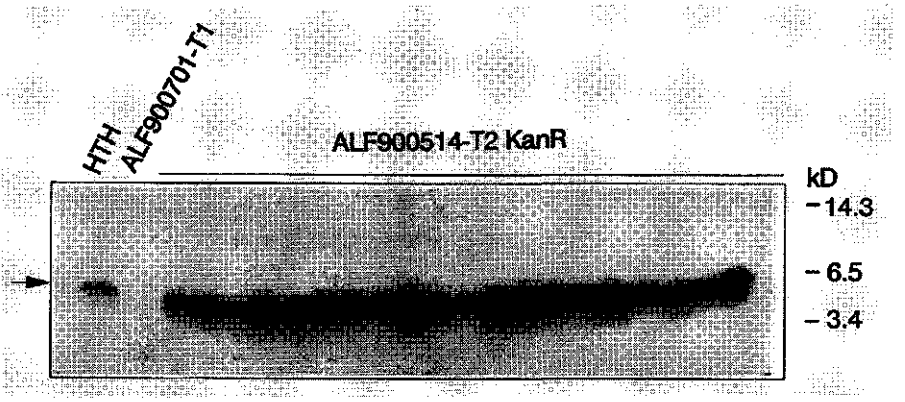


Figure 2. Immunoblot of leaf proteins from kanamycin resistant offspring plants of the primary transgenic tomato plant ALF900514-T_1, after incubation with the hordothionin antibody. Lane HTH, 50 ng purified hordothionin from barley endosperm; lane ALF900701-T1, 5 μg dilute sulphuric acid-extractable protein from leaves of control kanamycin resistant; lanes ALF900514-T2 KanR, proteins from leaves of kanamycin resistant ALF900514-T_2 plants. Proteins were separated by SDS-tricine-PAGE. Molecular weight markers are indicated at the right.

These experiments also indicated that the precursor protein was correctly processed into the 5 kD mature α-HTH protein. Plants homozygous for the NPTII and HTH gene were selected in the next generation (T_3) after selfing a number of kanamycin resistant progeny plants of ALF900514-T_1 (T_2-plants) and analysis of segregation. HTH expression was slightly elevated

in plants homozygous for the gene in comparison to hemizygous plants. In addition, HTH expression appeared to be uniform among all plants of all three generations tested. Protein gel blotting of intercellular washing fluids from leaves of plants exhibiting high HTH expression, indicated that the protein was not secreted into the apoplast (data not shown).

5.4.2 Analysis of resistance

Inoculation of T_3 plants of ALF900514-T_1, homozygous for the HTH and NPTII gene (ALF900514-T_3), with a bacterial suspension of *C. m.* subsp. *michiganensis*, resulted in typical wilting symptoms after 2 to 3 weeks (data not shown). This was comparable to control tomato plants suggesting that these plants had no enhanced resistance to this bacterium.

Determination of the growth of *X. c.* pv. *vesicatoria* in leaves of ALF900514-T_3, ALF900521-T_1, ALF900701-T_1 and Hawaii 7998, indicated that bacterial growth was not affected in ALF900514-T_3 and ALF900521-T_1 when compared to the control kanamycin resistant plant. However, growth was significantly reduced in leaves of Hawaii 7998 (Figure 3a). On the contrary, only control kanamycin resistant plants exhibited disease symptoms 13 days after spray-inoculation, whereas ALF900514-T_1 did not show spots (Figure 3b).

A

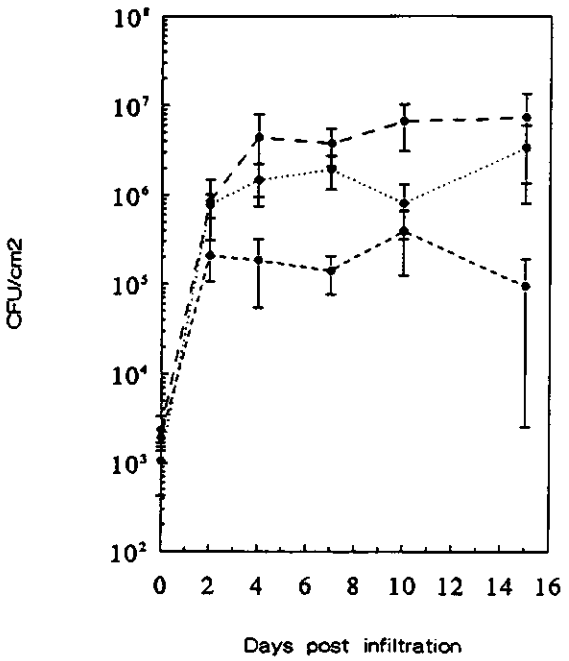
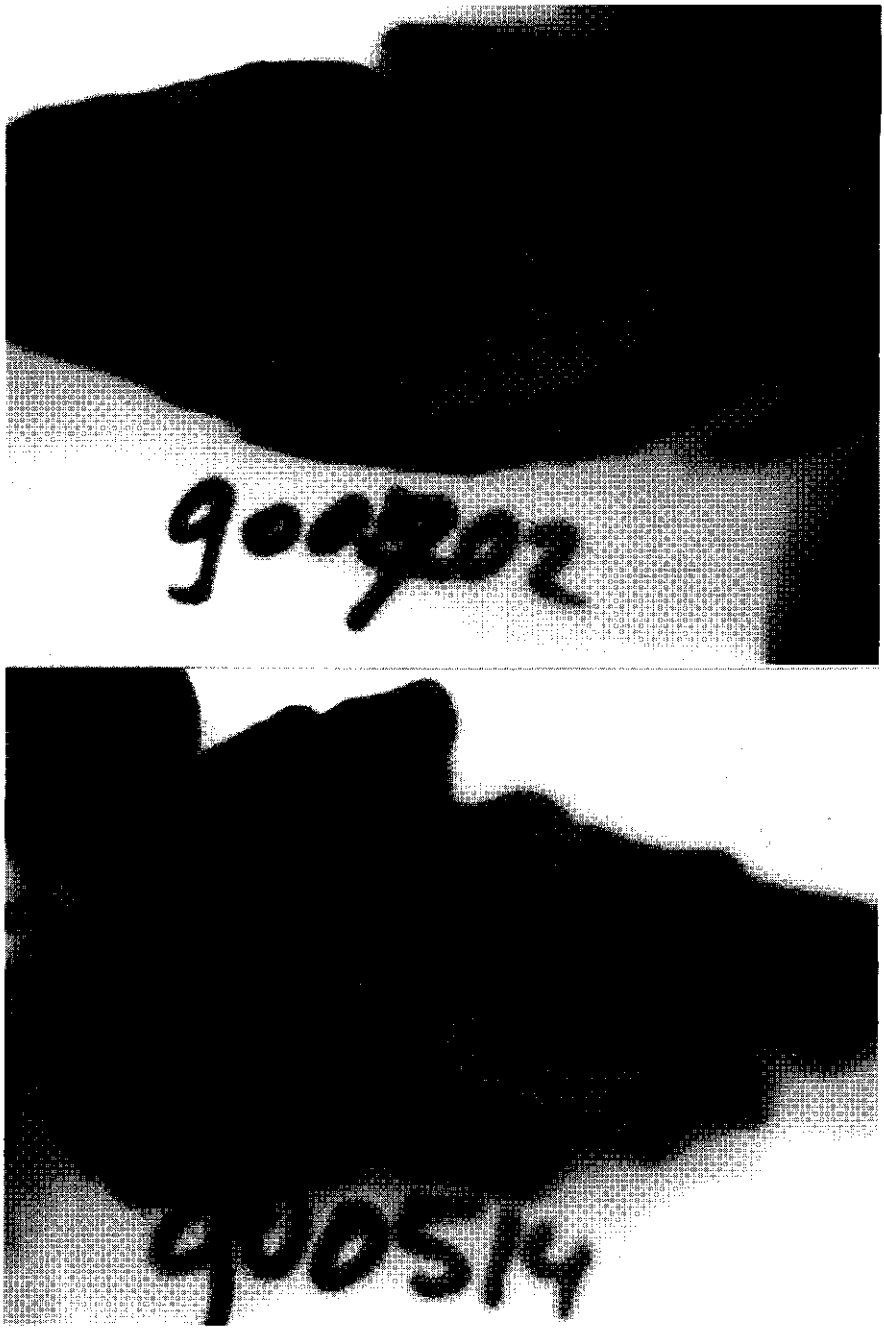


Figure 3. A. Populations of *Xanthomonas campestris* pv. *vesicatoria* in leaflets of tomato plants. Foliage-resistant Hawaii 7998 (---); transgenic tomato line ALF900514-T_3 showing high HTH expression (···) and control kanamycin resistant line ALF900701-T_1 (——). Leaves were infiltrated with a 1 x 10^5 CFU/ml bacterial suspension. Standard errors are indicated as vertical bars. B (left). Bacterial spot symptoms on transgenic tomato plants 13 days after spray-inoculation with a 1 x 10^8 CFU/ml bacterial suspension of *X. c.* pv. *vesicatoria*. ALF900702-T_1 (top) and ALF900514-T_1 (bottom). Typical spot symptoms were only observed on control plant and are indicated by arrows.

B



5.5 Discussion

The aim of our research was to investigate the feasibility of using the HTHs from barley endosperm for engineering bacterial disease resistance into plants. Previously we showed that only *C. m.* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus* and *X. c.* pv. *vesicatoria* were sensitive to HTHs *in vitro* [Florack *et al.*, 1990, 1993; chapter 2]. These bacteria cause diseases on tomato, pepper and potato. Expression studies in tobacco indicated that only gene constructs containing an amino-terminal signalpeptide resulted in HTH protein expression. The different precursor proteins appeared to be correctly processed into the 5 kD mature HTH, which was biologically active against *C. m.* subsp. *michiganensis in vitro*. Only gene constructs coding for the complete precursor protein harboring a signalpeptide and acidic-polypeptide, resulted in high HTH accumulation (chapter 4). Transgenic tobacco plants exhibiting high HTH expression levels were not evaluated for enhanced resistance because no bacteria pathogenic on tobacco had been found to be sensitive to HTH *in vitro*.

To study the potential of these proteins for engineering resistance, the gene construct resulting in highest HTH expression in tobacco, pC2α, was introduced in tomato. Our results indicated that the precursor protein was correctly processed into the 5 kD mature α-HTH in transgenic tomato and that high expression levels could be obtained using this gene construct in tomato. The transgenic plant exhibiting highest HTH expression, ALF900514-T_1, was chosen for the analysis of resistance. This plant segregated 3:1 for Kan^R:Kan^S upon selfing, and was made homozygous by selecting ALF900514-T_2 plants that did not segregate for the kanamycin resistance trait. The HTH gene appeared to be stably expressed in all three generations tested. Evaluation of these homozygous plants for resistance to *C. m.* subsp. *michiganensis*, indicated that these plants wilted at the same time as control tomato plants, following inoculation. In addition, analysis of the growth of *X. c.* pv. *vesicatoria* in leaves of this plant, indicated that this bacterium was not affected in growth rate compared to growth in leaves of control kanamycin resistant plants. On the contrary, growth of *X. c.* pv. *vesicatoria* was reduced in leaves of Hawaii 7998, which is in agreement with previous reports [Jones and Scott, 1986]. One explanation for these findings might be the intracellular location of the HTH which prevents a close contact between the antibacterial protein and these bacteria. *C. m.* subsp. *michiganensis* initially multiplies in the xylem vessels and subsequently spreads from these vessels to the adjacent intercellular spaces through phloem elements at advanced stages of pathogenesis [Wallis, 1977]. Spread of *X. c.* pv. *vesicatoria* is also restricted to the intercellular spaces [Stall and Cook, 1966]. Analysis of the intercellular washing fluids of these transgenic tomato plants exhibiting high HTH expression revealed that the HTH was not secreted into the apoplast. This is in agreement with previous results obtained for transgenic tobacco plants. In tobacco plants expressing different HTH precursor protein encoding genes, the HTH could also not be detected in the apoplast. In addition, protoplasts derived from these plants did not secrete HTH into the medium confirming an intracellular location of the protein. The observed difference between ALF900514-T_1 and ALF900701-T_1 when spray-inoculated with a bacterial suspension of *X. c.* pv. *vesicatoria* could be the result of a reduced infection frequency due to high HTH expression. Upon spray-inoculation, the bacterium infects leaves via the natural route, that is through stomata or wounds. Infection through wounds might lead to a close contact between the invading bacterium and the antibacterial HTH resulting in inhibition of the pathogen and hence reduced symptom development. Another explanation for the observed differences might be that the cell-wall texture has been altered by the expression of HTH. Confirmation of these results awaits further testing under conditions of high temperature and frequent rain, which are favorable for bacterial infection and spread. Field trials in Florida, where this pathogen causes one of the most destructive diseases of tomato, are in preparation.

Acknowledgements

D.F. was supported by a grant from the Dutch Programme committee Agricultural Biotechnology (PcLB). We are indebted to Jeffrey Jones and Lous van Vloten-Doting for helpful discussion, to Ab van Kammen for critical reading of the manuscript and helpful discussion and Pim Lindhout and Gerard Pet for the analysis of enhanced resistance to *C. m.* subsp. *michiganensis*. We want to thank Erik Dullaard, Dick Geurtsen and Peter de Jong for taking care of the transgenic tomato plants and Willem Geutjes and Egbert Kranen for technical support. Assays with plant pathogenic bacteria were performed with permission of the Netherlands Plant Protection Service at Wageningen.

5.6 References

De Wit PJGM, Spikman G: Evidence for the occurrence of race and cultivar-specific elicitors of necrosis in intercellular fluids of compatible and incompatible interactions of *Cladosporium fulvum* and tomato. Physiol Plant Pathol 21: 1-11 (1982).

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 Pathol 99: 259-268 (1993).

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 biotechnology in focus in the Netherlands, pp. 39-46. Pudoc, Wageningen (1990).

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).

Jones JB, Scott JW: Hypersensitive response in tomato to *Xanthomonas campestris* pv. *vesicatoria*. Plant Dis 70: 337-339 (1986).

Koornneef M, Hanhart C, Jongsma M, Toma I, Weide R, Zabel P, Hille J: Breeding of a tomato genotype readily accessible to genetic manipulation. Plant Sci 45: 201-208 (1986).

Mc Guire RG, Jones JB, Sasser M: Tween media for semiselective isolation of *Xanthomonas campestris* pv. *vesicatoria* from soil and plant material. Plant Dis 70: 887-891 (1986).

Murashige T, Skoog F: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497 (1962).

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).

Scott JW, Jones JB: Severity of bacterial spot (*Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye) on leaves and fruit of Florida grown tomato cultivars. Proc Fla State Hort Soc 97: 157-159 (1984).

Scott JW, Jones JB: Sources of resistance to bacterial spot in tomato. Hort Sci 21: 304-306 (1986).

Stall RE, Cook AA: Multiplication of *Xanthomonas vesicatoria* and lesion development in resistant and susceptible pepper. Phytopathol 56: 1152-1154 (1966).

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).

Van den Bulk RW, Lõffler HJM, Dons JJM: Effect of phytotoxic compounds produced by *Clavibacter michiganensis* subsp. *michiganensis* on resistant and susceptible tomato plants. Neth J Pl Path 95: 107-117 (1989).

Van Steekelenburg NAM: Resistance to *Corynebacterium michiganense* in tomato genotypes. Euphytica 34: 245-250 (1985).

Wallis FM: Ultrastructural histopathology of tomato plants infected with *Corynebacterium michiganense*. Physiol Plant Pathol 11: 333-342 (1977).

Weide R, Koornneef M, Zabel P: A simple, nondestructive spraying assay for the detection of an active kanamycin resistance gene in transgenic tomato plants. Theor Appl Genet 78: 169-172 (1989).

CHAPTER 6

Expression of giant silkmoth cecropin B genes in tobacco

Transgenic Research, in press (1994)

(with S. Allefs, R. Bollen, D. Bosch, B. Visser and W.J. Stiekema)

6.1 Abstract

Cecropin B is a small antibacterial peptide from the giant silkmoth *Hyalophora cecropia*. In this insect, cecropin B is processed from a larger precursor protein consisting of an amino-terminal signalpeptide, followed by two proline-containing dipeptides and the mature antibacterial cecropin B. This peptide was previously proven to be toxic *in vitro* for a large number of plant pathogenic bacteria [Nordeen *et al.*, 1992]. To reveal the potential of this peptide for engineering bacterial disease resistance into crops, several cecropin B gene constructs were made either for expression in the cytosol of plant cells or for secretion into the intercellular spaces of leaves. All constructs were cloned in a plant expression vector and introduced in tobacco by means of *Agrobacterium tumefaciens*. A cDNA-derived cecropin B gene construct lacking the amino-terminal signal peptide was poorly expressed in transgenic plants at the mRNA level whereas a full-length cDNA-derived construct containing the signalpeptide resulted in slightly higher cecropin B-mRNA levels. Highest cecropin B-mRNA expression was found in plants harboring a construct with a plant gene derived signalpeptide optimized for expression in solanaceous crops. In none of the transgenic plants the cecropin B peptide could be detected. In fact, the cecropin B peptide was shown to be highly unstable in tobacco cell extracts as the result of proteolytic degradation. Nevertheless, transgenic tobacco plants were evaluated for resistance to several plant pathogenic bacteria. Screening of selected transgenic tobacco plants for resistance to *Pseudomonas solanacearum*, the causal agent of bacterial wilt of many crops, indicated no significant differences in resistance between the cecropin B-mRNA expressing transgenic plants compared to control plants. In addition, growth of *P. syringae* pv. *tabaci*, the causal agent of bacterial wildfire, was not inhibited in selected transgenic tobacco plants. Both bacteria were reported to be highly susceptible to cecropin B *in vitro*. These experiments suggest that introduction and expression of cecropin B genes in tobacco does not result in enhanced resistance to plant pathogenic bacteria most likely because the peptide is rapidly degraded by plant endogenous proteases.

6.2 Introduction

Cecropins are a family of small ($M_r \approx 4000$), highly basic peptides which form an important key component in the immune-response of diverse insects [for reviews see Boman and Hultmark, 1987; Boman, 1991]. Cecropins were first identified as one of the major inducible peptides present in the hemolymph of immunized pupae of the giant silkmoth *Hyalophora cecropia* [Hultmark *et al.*, 1980; Steiner *et al.*, 1981]. A number of highly homologous cecropins were identified, all exhibiting lytic and antibacterial activity against several Gram-positive and Gram-negative bacteria *in vitro* [Hultmark *et al.*, 1982]. Cecropins belong to the family of the so-called pore-forming peptides [Ojcius and Young, 1991]. They are capable of forming ion-channels of approximately 4 nm in bacterial cell membranes by the adaptation of amphiphilic α-helices, resulting in leakage of cell-components and ultimately in death of bacteria [Christensen *et al.*, 1988]. The formation of pores does not require specific interactions with chiral receptors or enzymes [Christensen *et al.*, 1988; Wade *et al.*, 1990] which explains the broad antibacterial spectrum of cecropins.

Analysis of cDNA clones has shown that cecropins are made as precursor proteins of 62-64 amino acids [Van Hofsten *et al.*, 1985; Lidholm *et al.*, 1987]. Like most proteins destined for secretion [reviewed by Bednarek and Raikhel, 1992; Chrispeels, 1991; Rothman and Orci, 1992], cecropins are synthesized as precursors with an amino-terminal (N-terminal) signalpeptide, which is removed during (or shortly after) import into the endoplasmic

reticulum (ER) by a signalpeptidase [Boman *et al.*, 1989]. The signalpeptide of the cecropin B precursor has been shown to be functional in plants since it was capable of secreting proteins normally present in the cytosol, across the tobacco plasma membrane into the medium, when assayed in transient expression studies [Denecke *et al.*, 1990]. Experiments with chemically synthesized precursors of cecropin A and cecropin B in dog pancreas microsomes indicated that the signalpeptidase removed only the first 22 amino acids by cleaving the peptide bonds between Ala^{-5} and Ala^{-4} [Boman *et al.*, 1989]. The remaining propeptide was further processed in two steps by a dipeptidyl aminopeptidase, which subsequently removed the dipeptides Ala-Pro (residues -4 and -3) and Glu-Pro (residues -2 and -1) [Boman *et al.*, 1989]. *In vitro* translation of the cecropin A precursor indicated that the protein translocated both co-translationally (with the aid of signal recognition particle and ribosome) and post-translationally (without the involvement of ribonucleoparticles) in the presence of dog pancreas microsomes [Schlenstedt *et al.*, 1990]. All insect cecropins have an amidated carboxyl-terminus (C-terminus) which is the result of post-translational conversion of a glycine residue into the next-to-last α-carboxamide residue. Amidation is not essential for toxicity since synthetic cecropins with a C-terminal glycine residue with a free carboxyl group were shown to have similar toxicity levels [Boman *et al.*, 1989].

One of the cecropins, cecropin B, has gained a lot of attention from plant biotechnologists because of the high *in vitro* toxicity for a large number of plant pathogenic bacteria [Jaynes *et al.*, 1987; Nordeen *et al.*, 1992]. This renders the coding sequences of cecropin B into potentially powerful tools for engineering bacterial disease resistance into crops [Jaynes *et al.*, 1987]. Transgenic tobacco plants expressing Shiva-1, a cecropin B analogue with 46% sequence homology, were reported to have enhanced resistance to *Pseudomonas solanacearum*, a bacterial pathogen causing bacterial wilt, whereas plants expressing SB-37, an analogue with 95% sequence homology to cecropin B were not resistant [Jaynes *et al.*, 1993].

We report here the construction of three different cecropin B encoding sequences and the introduction and expression of these constructs in transgenic tobacco plants to study their potentiality for use in engineering bacterial disease resistance into plant crops. In addition, we present data on proteolytic degradation of a cecropin B peptide in tobacco cell extracts.

6.3 Materials and methods

6.3.1 Cecropin B plasmid constructions

pCP901 and pCP902, two partial cDNA clones isolated from *H. cecropia* encompassing the coding region for cecropin B, were kindly provided by H. Boman (University of Stockholm, Stockholm, Sweden). pCec1 was made by ligation of the *TaqI*/*Hin*cII fragment of pCP901 and the *Hin*cII/*Pst*I fragment of pCP902 [Van Hofsten *et al.*, 1985] in the Bluescribe pSK+ cloning vector (Stratagene Inc., La Jolla, USA). Modifications at the 5'- and 3'-regions of pCec1 to create pCec2 and pCec3, were introduced by the polymerase chain reaction (PCR) method. Oligonucleotides were made on an Applied Biosystems ABI380A DNA Synthesizer and purified by chromatography on oligonucleotide purification cartridges (OPC, Applied Biosystems, USA) according to the manufacturer.

For the construction of pCec2, an unique *Xho*I site was created at the 5'-end of pCec1 and an unique *Bam*HI site at the 3'-end, at the same time removing the 5' and 3' AT-rich untranslated regions. For the construction of pCec3, an artificial ATG initiation codon was created preceding the first lysine codon of the mature cecropin B part of pCec1 and an optimized translation initiation region according to Lütcke *et al.* [1987]. In addition, unique

*Eco*RI and *Hpa*I sites were created at the 5'- and an unique *Bam*HI site at the 3'-end of the gene, at the same time removing the first 26 amino acids of the cecropin B precursor and the AT-rich 3'-untranslated region. For the construction of pCec4 first a *Pst*I linker (5'-GCTGCAGC-3') was ligated in the *Hpa*I site of pCec3. The 5' *Eco*RI/*Pst*I fragment was subsequently replaced by the *Eco*RI/*Pst*I fragment of pBα, harboring the coding sequence of the signalpeptide of a barley leaf thionin [chapter 4; Florack *et al.*, 1994]. The reading frame was restored by *in vitro* mutagenesis according to Kunkel [1985] in such a way that the extra nucleotides introduced by this cloning step were removed and the putative signalpeptidase cleavage site was retained, using an oligonucleotide (5'-GTGCAAGTAGAAGGAAAATGG-AAGTCTTCAA-3'). All constructs were verified by sequence analysis using the dideoxy mediated chain termination method [Sanger *et al.*, 1977] on an Applied Biosystems ABI370A automated DNA Sequence Analyzer.

6.3.2 PCR amplification conditions, primers and amplified genes

Thermus aquaticus DNA polymerase (Amplitaq) was from Perkin Elmer-Cetus. PCR reactions were performed in 50 μl mixtures covered with mineral oil using the following buffer condition: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 5 μM of each dNTP, 20 pmoles of each primer, 1 ng of template and 1 unit *Taq*-polymerase. Amplification was for 30 cycles on a DNA Thermal Cycler 480 (Perkin Elmer-Cetus) as follows: 15 sec at 96°C, 30 sec at 60°C and 90 sec at 72°C.

A 238 basepair fragment containing the complete coding region of the cecropin B precursor was amplified from pCec1 as template, using (5'-GGGTACCGGGCCCCCCCC-3'), hybridizing to part of the polylinker of pSK+, and (5'-CGCGGATCCTATTATCCTAG-CGCTTTGGCTTC-3'), which is complementary to the 3'-end of the cecropin B gene and contained the *Bam*HI site. The amplified DNA was cloned as a *Xho*I/*Bam*HI fragment in pSK+ to render pCec2. A 140 basepair fragment containing the coding region of the mature cecropin B peptide was amplified from pCec1 as template using (5'-CTGGAATCGTTAACA-ATGAAATGGAAAGTCTTCAAGAAAATTG-3'), which is complementary to the mature cecropin B coding region of pCec1 and contained the *Eco*RI and *Hpa*I sites and the artificial ATG initiation codon (underlined), and the oligonucleotide harboring the *Bam*HI site mentioned above. This amplified DNA was cloned as an *Eco*RI/*Bam*HI fragment in pSK+ to render pCec3. Amplified fragments cloned in pSK+ were verified by sequence analysis.

6.3.3 Ligation in binary vector and plant transformation

For expression in plants, the cecropin B coding sequence of pCec2 was cloned as a *Xho*I/*Bam*HI fragment in the *Xho*I/*Bgl*II sites of pCPO5 [chapter 4; Florack *et al.*, 1994], resulting in pCPOC2. Those of pCec3 and pCec4 were cloned as *Hpa*I/*Bam*HI fragments in the *Sma*I/*Bgl*II sites of pCPO5 to render pCPOC3 and pCPOC4, respectively. Expression of the genes was controlled by a modified Cauliflower Mosaic Virus (CaMV) 35S promoter with doubled enhancer sequence [Kay *et al.*, 1985] and the nopaline synthase (nos) terminator sequence. The plant expression vectors were conjugated into *Agrobacterium tumefaciens* GV3101 (pMP90RK) [Koncz and Schell, 1986] by parental mating [Van Larebeke *et al.*, 1974] and used for transformation of tobacco (*Nicotiana tabacum* cv. Samsun NN) as described previously [chapter 4; Florack *et al.*, 1994].

6.3.4 Analysis of the expression of the cecropin B genes

Total RNA was isolated from young leaves (approximately 5 cm) of greenhouse-grown plants essentially as described [De Vries *et al.*, 1991]. 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 nitrocellulose membranes (GeneScreenPlus, New England Nuclear) for Northern analysis [Sambrook *et al.*, 1989] and hybridized with random primer ^{32}P-labelled DNA according to the manufacturer. The cecropin B coding sequences present in pCec2, pCec3 and pCec4 were used as probes. For quantitation, membranes were hybridized with a ribosomal DNA and a light harvesting complex protein gene probe (kindly provided by M. van Spanje).

Proteins were extracted from young leaves of greenhouse-grown plants. For small scale isolations, approximately 100 mg of leaf tissue was ground in an Eppendorf tube under liquid nitrogen and directly centrifuged at room temperature to obtain a total cellular extract, or incubated with a variety of buffers. Incubation was performed in 50 mM H_2SO_4 at room temperature and at 40°C [chapter 4; Florack *et al.*, 1994], in a buffer containing 80 mM Tris-HCl (pH 6.8), 2% SDS and 10% glycerol [Jaynes *et al.*, 1993], on ice and at room temperature, and in 15% (v/v) 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) at room temperature. After incubation debris was pelleted by centrifugation and the supernatant was transferred to a fresh tube. The amount of soluble protein was estimated by the method of Bradford [1976]. The intercellular washing fluids were isolated from young intact leaves [De Wit *et al.*, 1982], using water to *in vacuo* infiltrate the intercellular spaces.

Dot blot immuno-assays, SDS-tricine-polyacrylamide gelelectrophoresis (SDS-tricine-PAGE), blotting onto nitrocellulose membranes (BA 85, Schleicher and Schuell) and Western analysis using enhanced chemiluminescence (ECL, Amersham) and horse radish peroxidase-labelled secondary antibody were as described previously [chapter 4; Florack *et al.*, 1994]. Antiserum was raised in a rabbit against a synthetic cecropin B peptide, (NH_2-Lys-Trp-Lys-Val-Phe-Lys-Lys-Ile-Glu-Lys-Met-Gly-Arg-Asn-Ile-Arg-Asn-Gly-Ile-Val-Lys-Ala-Gly-Pro-Ala-Ile-Ala-Val-Leu-Gly-Glu-Ala-Lys-Ala-Leu-COOH) (M_r = 3836), purchased from the American Peptide Co., Inc. (Santa Clara, USA), which was coupled to bovine serum albumin using a water soluble carbodiimide prior to subcutaneous injection, to increase antigenicity [Deen *et al.*, 1990].

6.3.5 Analysis of cecropin B stability in tobacco cell extracts

Total cellular extracts and intercellular fluids of control tobacco plants were isolated as described above. A cytosolic extract was made from leaf mesophyl protoplasts of *in vitro* grown control tobacco plants by carefully pipetting the protoplasts through a yellow pipet tip, thereby destroying the plasma membrane integrity. Debris and microsomes were removed from the soluble cytoplasmic proteins by centrifugation at 14000 rpm for 10 min at 4°C [chapter 4; Florack *et al.*, 1994]. The stability of the cecropin B peptide was tested by mixing cytosol from approximately 5 x 10^4 protoplasts, 40 μl of intercellular fluids or total cellular extract, with known amounts of the peptide and incubation at room temperature, followed by freeze-drying at -50°C and analysis by protein gel blotting. The half-life ($t_{½}$) of cecropin B in these extracts was estimated by comparison of the results with those of known amounts of the peptide run on the same gel. The protease activities of these extracts were determined as described [Kitch and Murdock, 1986] using [^{14}C]methemoglobin as substrate. Similar amounts as used in the stability assays were incubated with 1.55 nmol substrate for 4 h in a total volume of 50 μl at 37°C, without pretreatment, after boiling for 15 min and after addition of HFP to a final concentration of 15% (v/v). Proteins in these mixtures were

precipitated with trichloroacetic acid and conversion of the substrate was measured by scintillation counting of the supernatants.

To establish what type of protease caused the degradation of cecropin B in intercellular fluid, a number of protease inhibitors were added to intercellular fluid, preincubated for 30 min at 30°C and tested in a similar assay as described above, except that incubation was carried out at 30°C. General inhibitors for serine proteases (phenylmethylsulfonyl fluoride [PMSF]; chymostatin), cysteine proteases (E-64), metalloproteases (EDTA; 1,10-phenantrolin) and aspartic proteases (pepstatin) were tested. These inhibitors were dissolved either in water or dimethyl sulfoxide (DMSO).

6.3.6 Growth of bacteria and inoculum

Pseudomonas solanacearum UW 213, a biovar 1, race 1 strain highly virulent on tobacco (A. Kelman, University Wisconsin, Madison, USA), was obtained from the Dutch Plant Protection Service (culture collection no. PD1456). Bacteria were grown overnight at 28°C on solid medium containing 10 g peptone, 1 g casamino acids, 1 g yeast extract, 5 g glucose and 15 g bacteriological agar per liter. Bacteria were suspended in phosphate buffered saline (PBS), centrifuged for 10 min at 3000 rpm and resuspended in PBS. The concentration of bacteria was estimated turbidimetrically at 595 nm (0.1 $OD_{595} \approx 1 \times 10^8$ colony forming units [CFU]/ml) and adjusted to 1×10^7 CFU/ml, which was used as inoculum. The exact amount of living bacteria in the inoculum was verified by dilution plating.

P. syringae pv. *tabaci* PTBR2.024, a highly virulent strain causing wildfire disease on tobacco and green beans [Engst and Shaw, 1992] was kindly provided by P. Shaw (University of Illinois-Champaign, Illinois, USA). Bacteria were grown overnight at 28°C on solid LB [Sambrook *et al.*, 1989] containing 100 mg/l rifampicillin and inoculum was prepared in 10 mM $MgSO_4$ as described above. Bacterial suspensions of 1.6×10^4 and 1.6×10^5 CFU/ml were used for infiltration of tobacco leaves.

6.3.7 Inoculation, disease assessment and statistical analysis

Primary T_1 transgenic plants were selected on the basis of highest cecropin B-mRNA expression, one per type of construct. These, in the next referred to as C2-8 (harboring the pCPOC2 gene construct), C3-22 (pCPOC3 gene construct) and C4-4 (pCPOC4 gene construct) and control untransformed Samsun NN plants were selfed. In experiment 1, seedlings of these plants (T_2 lines) were transplanted into 14 cm plastic pots in normal potting compost and placed in a growth chamber in randomized positions on a saucer. Plants approximately 15 cm in height, third or fourth leaf expanded, were inoculated with *P. solanacearum* essentially as described [Winstead and Kelman, 1952] by cutting the roots on one side of the plant, halfway the stem and wall of the pot with a sterile scalpel and slowly pouring 5 ml of the bacterial suspension onto the soil. Plants treated similarly but inoculated with PBS served as controls. Plants were incubated at 29°C at 12 h daylength and 90-100% relative humidity (RH) for five days to establish infection, and the soil was kept moist by watering onto the saucers. Temperature and RH were lowered after five days to 27°C and 70% respectively. Wilting was scored at intervals (7, 10, 13, 15, 17, 19, 22, 27 and 31 days after inoculation) using an arbitrary scale essentially as described [Martin and French, 1985] with minor modifications. Disease index of 0 = plant healthy; 1 = one leaf wilted; 2 = up to one third of plant wilted; 3 = up to two thirds of plant wilted and 4 = plant completely wilted.

In experiment 2, T_3-lines of the plants from experiment 1 that showed no wilting after 31 days, and progeny of the PBS-inoculated control plants, were tested similarly, except that in this experiment, wilting was scored 6, 9, 12, 16, 22, 29 and 36 days after inoculation.

For each experiment, disease ratings were converted prior to analysis, by calculating the area under the disease progress curve (ADPC-values) for each plant as described by Shaner and Finney [1977]. Analysis of variance (ANOVA) was carried out to analyze differences between the averaged ADPC-values of the selected transgenic lines, using the computer program GENSTAT (Rothamstead Experimental Station).

Plants from experiment 1 described above that showed no symptoms 31 days after inoculation, and control PBS-inoculated plants, were multiplied by making cuttings and used to establish growth of *P. syringae* pv. *tabaci* by infiltration into the underside of fully-expanded leaves using a syringe [Klement, 1963]. Infiltrated sectors were marked with a pencil and plants were kept at 28°C at 16 h daylength and 70% RH. At daily intervals, three punches with a diameter of 0.5 cm were collected from infiltrated sectors and macerated in 200 µl peptone-phosphate buffer [McGuire *et al.*, 1986]. Serial tenfold dilutions were made and appropriate dilutions were plated on solid LB supplemented with 100 mg/l rifampicillin in microplates. Bacteria were counted after incubation of plates for two days at 28°C.

6.4 Results

6.4.1 Cecropin B gene constructs and transformation

All gene constructs were cloned in pCPO5 [chapter 4; Florack *et al.*, 1994], a plant expression vector, under control of a double CaMV 35S promoter [Kay *et al.*, 1985]. The vectors containing the genes from pCec2, pCec3 and pCec4 were designated pCPOC2, pCPOC3 and pCPOC4 respectively. pCPOC2 coded for the full-length precursor cecropin B protein, pCPOC3 for the mature cecropin B peptide only, and pCPOC4 for the mature cecropin B peptide preceded by a leaf thionin-derived signalpeptide [chapter 4; Florack *et al.*, 1994]. pCPOC3 was aimed at studying cytosolic expression of cecropin B. pCPOC2 and pCPOC4, both encoding a cecropin B precursor harboring a signalpeptide, were aimed at studying secretion of cecropin B into the apoplast. Their nucleotide and amino acid sequences are depicted in Figure 1.

Transformation of tobacco resulted in 12 independent primary T_1 transgenic tobacco plants containing pCPOC2 (referred to as C2 transgenic plants), 25 containing pCPOC3 (C3 transgenic plants) and 15 containing pCPOC4 (C4 transgenic plants). All transgenic plants were grown to maturity in the greenhouse and were phenotypically normal and self-fertile, except for one C3 transgenic plant (C3-7), which had reduced internode-length and was male sterile.

6.4.2 Cecropin B expression in tobacco

Cecropin B-mRNA levels were estimated by Northern blot analysis of total RNA from all transgenic plants. Hybridization with cecropin B-specific probes indicated that all plants expressed the gene, however the level of cecropin B-mRNA varied depending on the construct used in the transformation event (Figure 2). Expression was highest in C4 transgenic plants containing the cecropin B construct with a plant signalpeptide, reaching a maximum of approximately 0.6% of polyadenylated RNA for C4-4 and an average of 0.2% (Figure 2, lanes pCPOC4). In C2 transgenic plants containing the full-length construct,

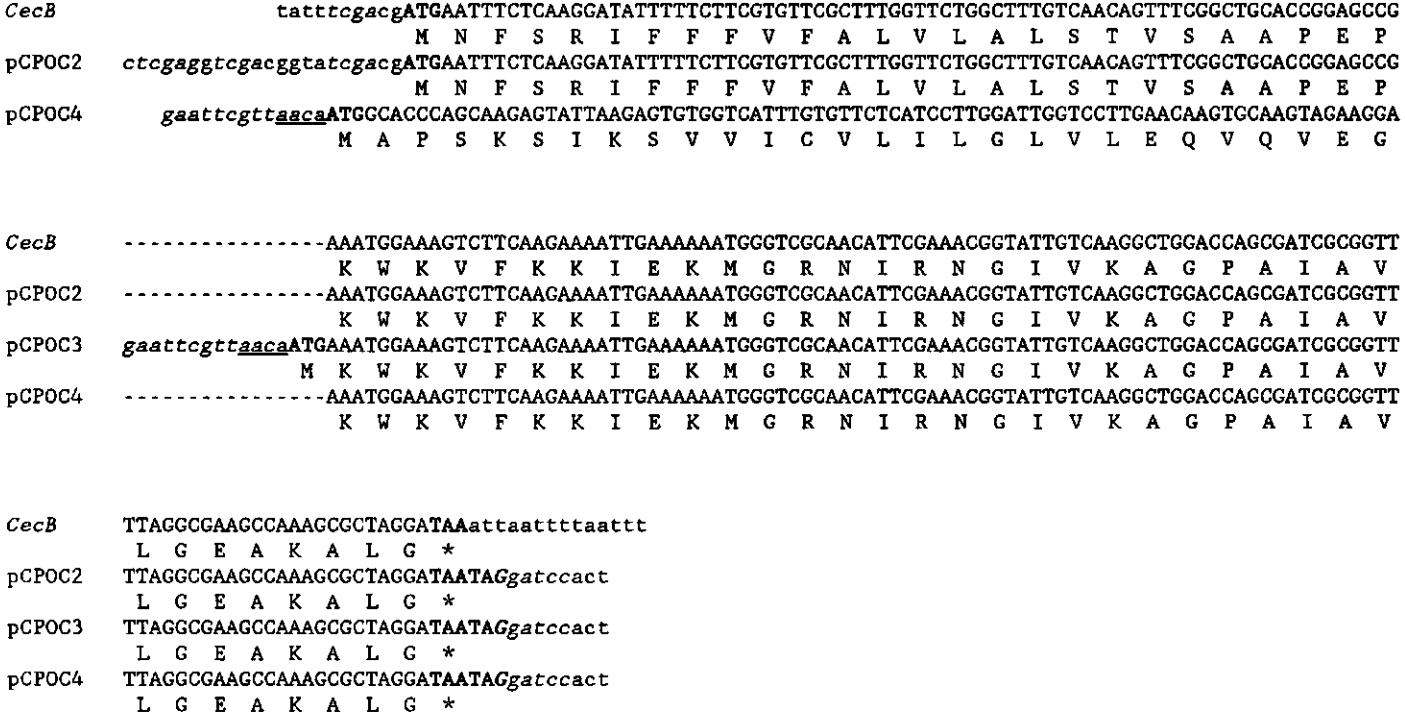


Figure 1. Nucleotide and amino acid sequences of cecropin B gene constructs pCPOC2, pCPOC3 and pCPOC4. The one-letter code for amino acids is used. For comparison, the corresponding sequences of the cecropin B gene, derived from pCP901 and pCP902 [Van Hofsten *et al.*, 1985], is shown on top. Translation initiation and stop codons are printed in boldface. Flanking *Taq*I, *Xho*I, *Bam*HI, *Eco*RI and *Hpa*I restriction sites are in italics and the optimized translation initiation region [Lütcke *et al.*, 1987] is underlined.

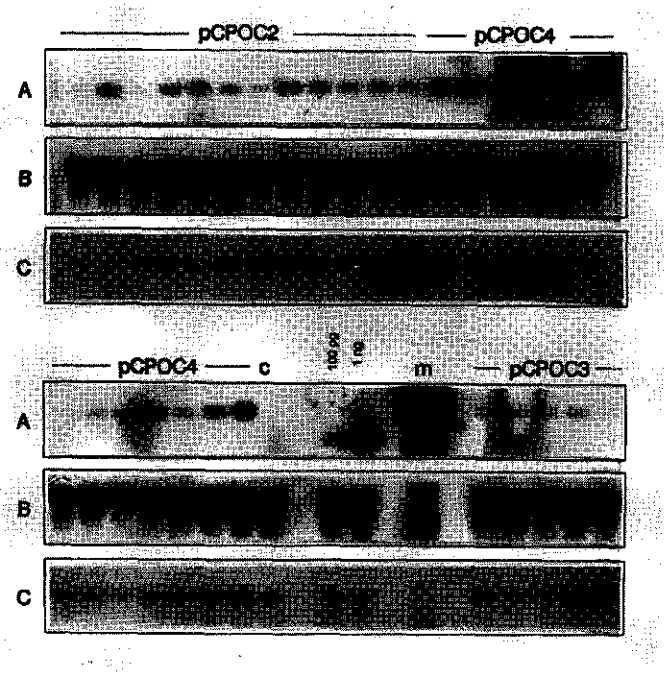


Figure 2. Autoradiographies of Northern blots containing total RNA of transgenic tobacco plants hybridized with different probes. Lanes pCPOC2, 10 μg of total RNA from transgenic plants harboring the pCPOC2 construct. Lanes pCPOC4, 10 μg of total RNA from plants harboring the pCPOC 4 construct, lane c, 10 μg of total RNA from control Samsun NN plant and lanes 100 pg and 10 ng, 10 μg of total RNA from control plant to which 100 pg and 1 ng of a DNA fragment containing the coding region of the pCPOC4 gene construct was added, respectively. Lane m, DNA size marker and lanes pCPOC3, 15 μg of total RNA from plants harboring the pCPOC3 construct. Membranes were hybridized with a cecropin B-specific probe (panel A), a ribosomal DNA probe (panel B) and a light harvesting complex protein gene probe (panel C).

cecropin B-mRNA levels were intermediate reaching a maximum of approximately 0.2% for C2-8 and an average of 0.1% (Figure 2, lanes pCPOC2). Expression was lowest in C3 transgenic plants carrying the mature cecropin B peptide coding region only, which exhibited very weak signals with a maximum expression level of 0.05% for C3-22 and an average of 0.02% (Figure 2, lanes pCPOC3).

Cecropin B peptide expression was studied of transgenic plants exhibiting the highest cecropin B-mRNA levels by dot blot immuno-assays and protein gel blotting using a polyclonal cecropin B antiserum. The detection limits of these assays, determined using a synthetic cecropin B peptide, was 30 to 50 ng and 10 ng respectively. No cecropin B peptide could be detected in the plants analyzed (data not shown) although especially in C4 transgenic plants considerable amounts of cecropin B-mRNA were present. This might be explained either by degradation of the cecropin B peptide during preparation of the samples or by a rapid turnover of the peptide in plant cells. This prompted us to investigate the stability of the cecropin B peptide in tobacco cell extracts.

6.4.3 Stability of cecropin B in tobacco cell extracts

Total cellular extract from ground leaf material, cytosol from isolated tobacco protoplasts and intercellular fluid from intact leaves, were prepared from control Samsun NN tobacco plants. To determine the stability of cecropin B in cellular fractions, known amounts of cecropin B were added and the mixtures were incubated at room temperature for various time periods. Analysis of samples by protein gel blotting indicated that the cecropin B peptide was degraded within seconds in intercellular fluid (Figure 3a, lanes 9 to 12). Degradation was prevented by boiling of extracts prior to adding cecropin B (Figure 3a, lanes 5 to 8), suggesting that it was degraded by a plant protease. Degradation was also prevented when incubations were performed in the presence of HFP (Figure 3a, lanes 1 to 4), the sole solvent in which cecropins fold into their biologically active conformation. The time period required for full degradation of synthetic cecropin B in cytosol and total cellular extract was longer (Table 1), suggesting that it is most likely degraded by extracellular proteases. In all cases, boiling of extracts prevented degradation of cecropin B. The half-life ($t_{½}$) was estimated by comparison of the results of degradation of cecropin B in the extracts with those of known amounts of cecropin B, after protein gel blotting, and was found to be less than 1 min for intercellular fluid (Figure 3a, compare lanes 9 to 12 with 13 and 14), 15 min for total extract and approximately 30 to 60 min for cytosolic extract. The half-lives determined in these experiments are listed in Table 1. The protease activities of these extracts were determined by measuring the degradation of a labelled generic substrate and indicated that degradation of the substrate was slow in all three extracts, suggesting low protease activities (Table 1). As expected, protease activity was reduced by boiling of the extracts prior to adding the substrate, and appeared to be lowest in extracts containing HFP (Table 1), probably the result of denaturation of proteases (or substrate) by HFP.

Further characterization of the cause of cecropin B degradation in intercellular fluid was carried out using specific protease inhibitors. Only in the presence of chymostatin (inhibitor of chymotrypsin, a serine protease) and PMSF (inhibitor of serine and cysteine proteases), degradation of cecropin B by intercellular fluid was reduced (Figure 3b). On the contrary, degradation of cecropin B could not be inhibited by the addition of E-64, pepstatin, 1,10-phenantrolin and EDTA (Figure 3b).

Table 1. Half-lives of cecropin B peptide in the three extracts and protease activities of the extracts without pretreatment, after boiling and after the addition of HFP as calculated from the conversion of [^{14}C]methemoglobin after incubation at 37°C for 4 h

Extract	Treatment	Half-life (min)	Conversion[1]	Relative activity[2]
Cytosol	no	30	0.25	100
Cytosol	boiled	> 30	0.18	72
Cytosol	+ HFP	> 240	0.08	32
Intercellular fluid	no	< 1	0.56	100
Intercellular fluid	boiled	> 30	0.16	29
Intercellular fluid	+ HFP	> 240	0.09	16
Total	no	15	0.76	100
Total	boiled	> 30	0.14	18
Total	+ HFP	> 240	0.06	8

[1] Protease activity is calculated by estimation of the release of[^{14}C]-met from [^{14}C]-methemoglobin after incubation by scintillation counting

[2] Relative activity is depicted per type of extract and is set at 100% for untreated extract

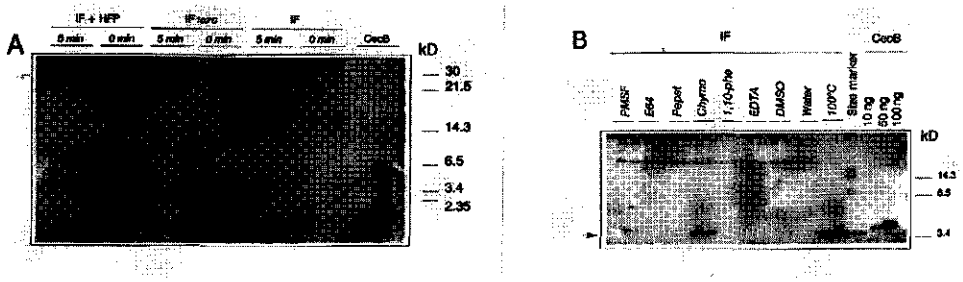


Figure 3. Stability of cecropin B in intercellular fluid of tobacco leaves. Proteins were separated by SDS-tricine-PAGE and protein gel blotting was performed using a cecropin B antibody. A. Stability in intercellular fluid. Lanes 1 and 2, intercellular fluid + 15% HFP + 100 ng or 300 ng cecropin B respectively, incubated for 5 min at room temperature (RT); lanes 3 and 4, intercellular fluid + 15% HFP + 100 ng and 300 ng cecropin B respectively, directly frozen and freeze-dried; lanes 5 and 6, intercellular fluid boiled for 15 min before adding 100 or 300 ng cecropin B respectively and incubated for 5 min at RT; lanes 7 and 8, intercellular fluid boiled for 15 min before adding 100 or 300 ng cecropin B respectively, frozen directly and freeze-dried; lanes 9 and 10, intercellular fluid + 100 or 300 ng cecropin B respectively, incubated for 5 min at RT; lanes 11 and 12, intercellular fluid + 100 or 300 ng cecropin B respectively, directly frozen and freeze-dried; lanes 13 and 14, 100 and 300 ng cecropin B respectively. Molecular weight markers are indicated at the right.
B. Inhibition of degradation by protease inhibitors. Intercellular fluid was treated for 30 min with different protease inhibitors (indicated above the lanes), after which the substrate (cecropin B) was added. Incubation was continued for 15 min (first lane of each pair) to 60 min (second lane) at 30°C. Degradation was terminated by the addition SDS to a final concentration of 1%, followed by incubation for 30 min at 40°C. As controls, intercellular fluid was incubated with DMSO which is the solvent of many of the protease inhibitors tested; no inhibitor was added (water) or intercellular fluid was boiled for 10 min prior to adding cecropin B (100°C). The position of cecropin B is marked by an arrow at the left.

6.4.4 Analysis of resistance

Although we could not detect the cecropin B peptide in any of the transgenic tobacco plants expressing the different cecropin B gene constructs at the mRNA level, plants having highest cecropin B-mRNA concentrations were evaluated for bacterial disease resistance. In two experiments resistance to the bacterial pathogen *P. solanacearum* was evaluated. This phytopathogen infects plants via wounds on roots and spreads through xylem vessels, which are subsequently plugged with bacterial slime and aggregates resulting in bacterial wilt [Goto, 1992]. In experiment 1, 23 progeny plants of C2-8, C3-22 and C4-4, which were selected on the basis of highest cecropin B-mRNA levels, were tested. In experiment 2, 50 progeny plants of C2-8-6, C3-22-6 and C3-22-22, which were the sole survivors of experiment 1, 31

days after inoculation, were tested. Analysis of segregation for kanamycin resistance indicated that C2-8-6 was homozygous (Kan^R : Kan^S = 66 : 0) and that C3-22-6 (Kan^R : Kan^S = 61 : 4) and C3-22-22 (Kan^R : Kan^S = 83 : 4) were probably hemizygous for more than one locus. Although both inoculation experiments were performed under similar conditions, disease development was much slower in the second experiment resulting in more plants showing no symptoms (Table 2). In these experiments, no significant differences for the mean ADPC-values of transgenic and control lines were found (Table 2).

Table 2. Number of plants with and without wilting symptoms at 31 (experiment 1) and 36 (experiment 2) days after inoculation with *Pseudomonas solanacearum* UW 213, and mean areas under the disease progress curves (ADPC) values of 6 transgenic and a control tobacco line in two experiments

Line	no wilting	wilting	ADPC-value
EXPERIMENT 1			
N. tabacum Samsun NN	0	23	0.68
N. tabacum C2-8	1	22	0.64
N. tabacum C3-22	2	21	0.62
N. tabacum C4-4	0	23	0.75
SED[1] (DF 88) = 0.06			
EXPERIMENT 2			
N. tabacum Samsun NN	19	31	0.38
N. tabacum C2-8-6	12	38	0.46
N. tabacum C3-22-6	10	40	0.49
N. tabacum C3-22-22	10	40	0.41
SED (DF 194) = 0.07			

[1] Abbreviations: SED, standard error of differences of means; DF, degrees of freedom

In vivo growth of *P. syringae* pv. *tabaci*, which multiplies in the intercellular spaces of leaves and produces typical chlorotic halos in compatible interactions [Stewart, 1971], was determined in C2-8-6, C3-22-6, C3-22-22 and control Samsun NN plants by infiltration of a bacterial suspension into leaves and determination of bacterial growth. In Figure 4 growth curves obtained using the lowest inoculum density are shown together with the standard deviations. No differences were seen between control and selected transgenic plants. Similar results were obtained for tenfold higher inoculum density (data not shown). Typical chlorotic halos appeared three days after infiltration with the 1.6×10^5 CFU/ml bacterial suspension on all plants.

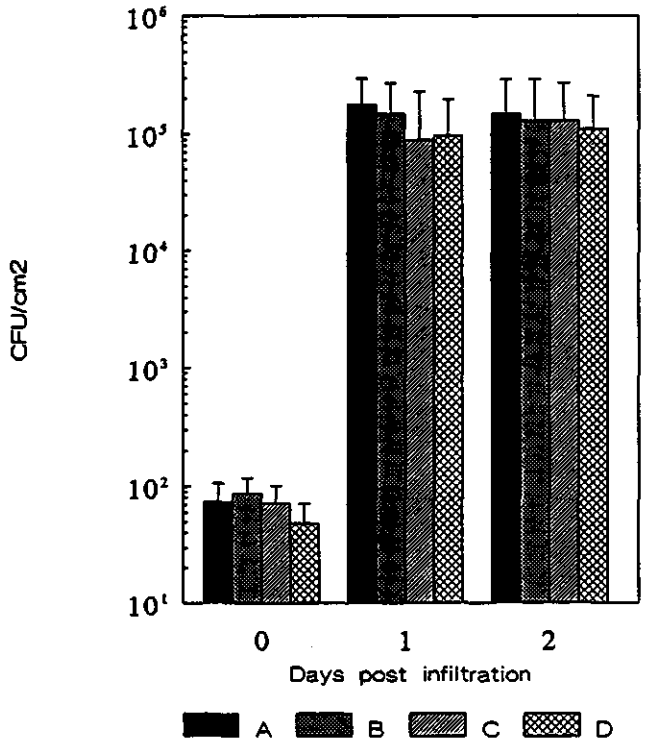


Figure 4. Growth of *Pseudomonas syringae* pv. *tabaci* PTBR2.024 in leaves of tobacco plants. Leaves of five plants each (multiplied by cutting) of selected transgenic plants C2-8-6 (A), C3-22-6 (B) and C3-22-22 (C), all three exhibiting high cecropin B-mRNA levels, and control (D) plants were infiltrated with a 1.6 x 10^4 CFU/ml bacterial suspension and the number of CFU/cm^2 was determined at 0, 1 and 2 days *post* inoculation by grinding leaf discs of known diameter in a buffer and plating appropriate tenfold dilutions in selective medium. Standard errors are indicated by vertical bars.

6.5 Discussion

Prerequisites for application of cecropin B genes in engineering bacterial disease resistance into crops, are expression of the gene and synthesis of the peptide to a level sufficient to kill the pathogen and targeting to the compartment where the pathogen resides. Different cecropin B constructs were made aimed at expression in the cytoplasm and secretion into the apoplast. All were placed under control of a doubled CaMV 35S promoter [Kay *et al.*, 1985], which directed high ß-glucuronidase expression in leaves, stems and roots of tobacco [Jefferson *et al.*, 1987], the tissues invaded by the pathogens under study. Previously, this promoter was also successfully used to drive high level expression of the antibacterial hordothionin in tobacco leaves [chapter 4; Florack *et al.*, 1994]. Both hordothionin-mRNA and protein reached levels up to 0.5-1% of total mRNA and soluble protein respectively in these plants. Hordothionin-mRNA expression levels in tobacco depended slightly upon the construct in contrast to cecropin B-mRNA expression levels which depended heavily upon the construct. Levels up to 0.6% of total polyadenylated RNA were found in transgenic plants containing the pCPOC4 gene construct (C4 transgenic plants). In the pCPOC4 construct, the coding sequence of the mature cecropin B is fused to the coding sequence of a leaf thionin

signalpeptide. This sequence was chemically synthesized and designed to obtain high level expression in solanaceous crops by adaptation of the codon usage and optimization of the translation initiation region [chapter 3]. It was made to obtain secretion of the protein into the apoplast and its presence apparently positively influences cecropin B-mRNA expression. Cecropin B-mRNA expression was lowest in plants containing pCPOC3 which lacked a signalpeptide coding sequence, although the DNA sequence directly upstream the translation initiation region and downstream of the coding region of this gene and of pCPOC4 were identical. One explanation might be that the presence of a signalpeptide coding sequences in pCPOC2 or pCPOC4 increases the half-life of the mRNA's, suggesting coupling between protein secretion, translation and mRNA stability. Instability of the messengers as the result of AT-rich repeats in the cecropin B coding region [Van Hofsten *et al.*, 1985; see Figure 1B], which in plants could lead to premature transcription termination as shown for *Bacillus thuringiensis* genes [Murray *et al.*, 1991; Perlak *et al.*, 1991], is unlikely because only one distinct messenger of expected size is seen on Northern blots.

Although considerable amounts of cecropin B-mRNA were present in most C4- and some of the C2-transgenic plants, no cecropin B peptide could be detected in the plants analyzed. The observation that various plant cell extracts rapidly degraded a synthetic cecropin B peptide, whereas boiling of the extracts and addition of specific inhibitors, prevented degradation, supports the idea that cecropin B is degraded by plant proteases. Most likely the peptide is degraded by a serine protease since degradation could only be inhibited by chymostatin and to a lesser extent by PMSF, whereas it could not be inhibited by E-64. This sensitivity to proteases might be caused by the random conformation cecropin B adopts in a hydrophilic environment [Steiner, 1982; Holak *et al.*, 1988]. Cecropin B only folds into its characteristic amphipathic conformation when the peptide interacts with a bacterial membrane [Christensen *et al.*, 1988] or in a solution resembling the characteristics of such a membrane, like HFP [Steiner, 1982; Holak *et al.*, 1988]. Addition of HFP to extracts inhibited protease activity in general. Extraction of proteins from leaves of transgenic tobacco plants in the presence of HFP, did not result in recovery of detectable amounts of cecropin B, although degradation of synthetic cecropin B in a reconstitution experiment was prevented by the addition of HFP and large amounts of leaf material of transgenic plants were analyzed. These results suggest a high turnover of the cecropin B peptide in transgenic tobacco cells. SB-37, an analogue with 95% sequence homology to cecropin B, could also not be detected in transgenic tobacco plants by Western blotting [Jaynes *et al.*, 1993]. On the contrary, minor amounts of Shiva-1, a cecropin B analogue with 46% sequence homology, could be detected in transgenic tobacco plants. This might be the result of the different amino acid composition, resulting in a lesser susceptibility to protease degradation, although Shiva-1 has similar aromatic amino acids which are most likely the amino acids cleaved by serine proteases.

Inoculation experiments with plant pathogenic bacteria did not show significant differences in resistance, or growth of bacteria in the intercellular spaces, between transgenic and control plants, which is in agreement with our expectation of a rapid turnover of cecropin B in plant cell extracts. In addition, the CaMV 35S promoter used in our experiments to drive expression of the different genes, was previously reported to have highest activity in the phloem tissues along the inside and outside of the vascular ring, whereas one of the pathogens we tested, *P. solanacearum* invades the xylem vessels [Goto, 1992] and therefore might not come into close contact with the cecropin B peptide before the onset of tissue maceration. Transgenic tobacco plants expressing SB-37 had no resistance to *P. solanacearum* [Jaynes *et al.*, 1993], which is in agreement with our results.

In conclusion, cecropin B-mRNA expression in transgenic tobacco depended heavily upon the construct used in the transformation event. Despite the presence of considerable amounts of cecropin B-mRNA in plants, no cecropin B peptide could be detected, not even

after disruption of membranes by treatment with SDS or dilute sulphuric acid, or when extracts were made in the presence of protease inhibitors and HFP. Moreover, the results presented in this chapter provide experimental evidence that the antibacterial cecropin B peptide is highly unstable in tobacco cell extracts and is degraded by (a) plant endogenous protease(s), most likely a serine protease. The putative low (undetectable) steady state levels of cecropin B were insufficient to protect plants to bacterial infections with *P. solanacearum* UW213 and did not inhibit growth of *P. syringae* pv. *tabaci* PTBR2.024 *in vivo*. These findings make a successful application of cDNA-derived cecropin B sequences and closely resembling analogues for engineering resistance into solanaceous crops highly unlikely.

Acknowledgements

D.F. was supported by a grant from the Dutch Programmatic Business orientated Technology Stimulation fund (PBTS). We would like to thank Hans Boman for the gift of pCP901 and pCP902, Martin van Spanje for the gift of probes, Paul Shaw for the gift of *P. syringae* pv. *tabaci* PTBR2.024, Wim Dirkse for sequencing and Ab van Kammen for critical reading of the manuscript. We also would like to thank Johan Hulsman and Hans Janssen for taking care of the transgenic plants, Willem Geutjes and Egbert Kranen for technical support, Maarten Jongsma for the analysis of protease activity and Paul Keizer for helpful discussion on statistics and Elly Munsterman for the analysis of growth of *P. syringae* pv. *tabaci* in plants. All experiments with *Pseudomonas* spp. were performed with permission of the Dutch Plant Protection Service.

6.6 References

Bednarek SY, Raikhel NV: Intracellular trafficking of secretory proteins. Plant Mol Biol 20: 133-150 (1992).

Boman HG, Boman IA, Andreu D, Li Z-q, Merrifield RB, Schlenstedt G, Zimmerman R: Chemical synthesis and enzymic processing of precursor forms of cecropins A and B. J Biol Chem 264: 5852-5860 (1989).

Boman HG: Antibacterial peptides: key components needed in immunity. Cell 65: 205-207 (1991).

Boman HG, Hultmark D: Cell-free immunity in insects. Ann Rev Microbiol 41: 103-126 (1987).

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).

Chrispeels MJ: Sorting of proteins in the secretory system. Annu Rev Plant Physiol Plant Mol Biol 42: 21-53 (1991).

Christensen B, Fink J, Merrifield RB, Mauzerall D: Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. Proc Natl Acad Sci USA 85: 5072-5076 (1988).

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).

Denecke J, Botterman J, Deblaere R: Protein secretion in plant cells can occur via a default pathway. Plant Cell 2: 51-59 (1990).

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).

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 Pathol 21: 1-11 (1982).

Engst K, Shaw PD: Identification of a *lysA*-like gene required for tabtoxin biosynthesis and pathogenicity in *Pseudomonas syringae* pv. *tabaci* strain PTBR2.024. Mol Plant-Microbe Inter 5: 322-329 (1992).

Florack DEA, Dirkse WG, Visser B, Heidekamp F, Stiekema WJ: 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. Plant Mol Biol 24: 83-96 (1994).

Goto M: Fundamentals of bacterial plant pathology. Academic Press, Inc., San Diego (1992).

Holak TA, Engström Å, Kraulis PJ, Lindeberg G, Bennich H, Jones TA, Gronenborn AM, Clore GM: The solution conformation of the antibacterial peptide cecropin A: a nuclear magnetic resonance and dynamical simulated annealing study. Biochem 27: 7620-7629 (1988).

Hultmark D, Steiner H, Rasmusson T, Boman HG: Insect immunity: purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. Eur J Biochem 106: 7-16 (1980).

Hultmark D, Engström Å, Bennich H, Kapur R, Boman HG: Insect immunity: isolation and structure of cecropin D and four minor antibacterial components from cecropia pupae. Eur J Biochem 127: 207-217 (1982).

Jaynes JM, Nagpala P, Destéfano-Beltrán L, Huang JH, Kim JH, Denny T, Cetiner S: Expression of a Cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum*. Plant Sci 89: 43-53 (1993).

Jaynes JM, Xanthopoulos KG, Destéfano-Beltrán L, Dodds JH: Increasing bacterial disease resistance utilizing antibacterial genes from insects. BioEssays 6: 263-270 (1987).

Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907 (1987).

Kay R, Chan A, Daley M, Mc Pherson J: Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236: 1299-1302 (1985).

Kitch LW, Murdock LL: Partial characterization of a major gut thiol proteinase from larvae of *Callosobruchus maculatus* F. Arch Insect Biochem Physiol 3: 561-575 (1986).

Klement Z: Rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature 199: 299-300 (1963).

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).

Kunkel TA: Rapid and efficient site-directed mutagenesis without phenotypic selection. Proc Natl Acad Sci USA 82: 488-492 (1985).

Lidholm D-A, Gudmundsson GH, Xanthopoulos KG, Boman HG: Insect immunity: cDNA clones coding for the precursor forms of cecropins A and D, antibacterial proteins from *Hyalophora cecropia*. FEBS Lett 226: 8-12 (1987).

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).

Martin C, French ER: Bacterial wilt of potato: *Pseudomonas solanacearum*. Technical information bulletin 13. International Potato Center, Lima, Peru 16 pp (1985).

McGuire RG, Jones JB, Sasser M: Tween media for semiselective isolation of *Xanthomonas campestris* pv. *vesicatoria* from soil and plant material. Plant Disease 70: 887-891 (1986).

Murray EE, Rocheleau T, Eberle M, Stock C, Sekar V, Adang M: Analysis of unstable RNA transcripts of insecticidal crystal protein genes of Bacillus thuringiensis in transgenic plants and electroporated protoplasts. Plant Mol Biol 16: 1035-1050 (1991).

Nordeen RO, Sinden SL, Jaynes JM, Owens LD: Activity of cecropin SB37 against protoplasts from several plant species and their bacterial pathogens. Plant Sci 82: 101-107 (1992).

Ojcius DM, Young JD-E: Cytolytic pore-forming proteins and peptides: is there a common structural motif? TIBS 16: 225-229 (1991).

Perlak FJ, Fuchs RL, Dean DA, McPherson JM, Owens LD: Modification of the coding sequence enhances plant expression of insect control protein genes. Proc Natl Acad Sci USA 88: 3324-3328 (1991).

Rothman JE, Orci L: Molecular dissection of the secretory pathway. Nature 355: 409-415 (1992).

Sambrook J, Fritsch EF, Maniatis T: Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989).

Sanger F, Nicklen S, Coulson AR: DNA sequencing with the chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467 (1977).

Schlenstedt G, Gudmundsson GH, Boman HG, Zimmermann R: A large presecretory protein translocates both cotranslationally, using signal recognition particle and ribosome, and post-translationally, without these ribonucleoparticles, when synthesized in the presence of mammalian microsomes. J Biol Chem 23: 13960-13968 (1990).

Shaner G, Finney RE: The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathol 67: 1051-1056 (1977).

Steiner H: Secondary structure of the cecropins: antibacterial peptides from the moth *Hyalophora cecropia*. FEBS Lett 137: 283-287 (1982).

Steiner H, Hultmark D, Enström Å, Bennich H, Boman HG: Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292: 246-248 (1981).

Stewart W: Isolation and proof of structure of wildfire toxin. Nature 229: 174-178 (1971).

Van Hofsten P, Faye I, Kockum K, Lee J-Y, Xanthopoulos KG, Boman IA, Boman HG, Engström Å, Andreu D, Merrifield RB: Molecular cloning, cDNA sequencing, and chemical synthesis of cecropin B from *Hyalophora cecropia*. Proc Natl Acad Sci USA 82: 2240-2243 (1985).

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 (1977).

Wade D, Boman A, Wåhlin B, Drain CM, Andreu D, Boman HG, Merrifield RB: All-D amino acid-containing channel-forming antibiotic peptides. Proc Natl Acad Sci USA 87: 4761-4765 (1990).

Winstead NN, Kelman A: Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathol 42: 628-634 (1952).

CHAPTER 7

General discussion

7.1 Plant pathogenic bacteria and pathogenesis

The interactions between bacterial pathogens and plants can be severalfold: no interaction is observed, there is an interaction beneficial for both, there is a compatible interaction leading to bacterial growth and symptom development or an incompatible interaction resulting in the absence of observable disease symptoms. In a compatible interaction the phytopathogen can invade the plant resulting in typical disease symptoms. The primary infection site, spreading of the pathogen and disease symptom development which ultimately lead to a decrease of crop yield can differ greatly. In the following I will focus only on bacterial pathogens causing serious decrease of crop yield in the economically important tobacco, tomato and potato, which are our model crops. The most important ones are listed in Table 1.

Table 1. Plant pathogenic bacteria of solanaceous crops

Bacteria	Gram +/-[1]	Disease name
Clavibacter michiganensis subsp. *michiganensis*	+	Bacterial canker on tomato
Clavibacter michiganensis subsp. *sepedonicus*	+	Ring rot on potato
Erwinia carotovora subsp. *atroseptica*	-	Soft rot and/or blackleg on potato
Erwinia carotovora subsp. *carotovora*	-	Soft rot and/or blackleg on potato
Erwinia chrysanthemi	-	Soft rot and/or blackleg on potato
Pseudomonas solanacearum	-	Bacterial wilt on numerous crops
Pseudomonas syringae pv. *tabaci*	-	Bacterial wildfire on tobacco
Pseudomonas syringae pv. *tomato*	-	Bacterial speck on tomato
Xanthomonas campestris pv. *vesicatoria*	-	Foliage and fruit spot on tomato

[1] Reaction to Gram staining

Clavibacter michiganensis subsp. *michiganensis* infects tomato plants via foliar trichomes, cuttings or wounds and spreads from the xylem vessels to the adjacent intercellular spaces and phloem elements at advanced stages of pathogenesis [Wallis, 1977]. *C. michiganensis* subsp. *sepedonicus* infects potato plants primarily through tuber wounds, but also through wounds in stems, roots, stolons and other plant parts [Stefani, 1989]. Subsequently, vessels, xylem parenchyma and adjacent tissue are invaded. *Erwinia carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* infect potato plants and tubers in the field but also after harvest and during storage, through stomata, wounds and lenticels [Fox *et al.*, 1972]. These pathogens spread through tubers mainly via the intercellular spaces of the storage parenchyma, but can also invade xylem and phloem elements resulting in tuber soft rot and/or blackleg. Tuber soft rot is characterized by maceration of the parenchymatous tissue and blackleg by stem rot [Elphinstone, 1987]. *Pseudomonas solanacearum* causes a devastating disease on numerous plant species including potato, tomato and tobacco and infects plants via infested soil [Kelman, 1953]. The pathogen enters through wounds occurring during cultivation or natural growth of secondary roots, or through injuries caused by the root-knot and other nematodes [Martin and French, 1985]. After invasion of the roots, the pathogen spreads through xylem vessels which are rapidly plugged with bacterial slime and cell aggregates eventually causing blocking of water conductance resulting in wilting [Goto, 1992]. *P. syringae* pv. *tabaci* infects plants through stomata and subsequently multiplies in the intercellular spaces where it produces a toxin (tabtoxin) causing an elevated ammonium level in leaves resulting in the typical chlorotic halos [Stewart, 1971]. *P. syringae* pv. *tomato* also infects plants through stomata and wounds and is restricted to the intercellular spaces, but this bacterium produces proteases which upon degradation of plant proteins, result in elevated ammonium levels leading to chlorotic halos [Goto, 1992]. *X.*

campestris pv. *vesicatoria* infects tomato and pepper plants similarly via stomata or wounds and subsequent spread is also restricted to the intercellular spaces [Stall and Cook, 1966]. At advanced stages of pathogenesis whole leaves are invaded by the pathogen resulting in necrosis of the infected tissue.

Traditional plant breeding, the introduction of desirable traits by means of sexual crosses, has not been very successful with respect to achieving bacterial disease resistant plants. Resistant forms of the plant may not exist, or may be difficult to find for each new strain (or race) of the parasite which arises. Such resistances may be polygenic hampering an easy introgression and if the resistance is monogenic (gene-for-gene relationship), the pathogen often has evolved strategies for overcoming such resistance. For these reasons, we have chosen an alternative strategy to obtain bacterial disease resistant plants.

7.2 Application of hordothionins for engineering resistance

A novel approach in producing resistance to plant parasites, is the application of genetic engineering technology. Several possibilities can be thought of and in this thesis one of these, the feasibility of the introduction of genes coding for antibacterial proteins was examined.

Our first choice to use thionin encoding genes was based on the reported toxicity of the thionin from wheat endosperm (purothionin) for plant pathogenic bacteria *in vitro* [Fernandez de Caleya *et al.*, 1972] and the presumption that expression of plant-derived genes in other plant species (solanaceous crops) would not cause serious problems. Because of the availability of detailed molecular data, such as knowledge of the primary amino acid sequence and cDNA sequences, we chose to investigate the feasibility of the thionins from barley endosperm (hordothionins) for engineering. To establish toxicity of these hordothionins for plant pathogenic bacteria, these proteins were isolated from barley endosperm [chapter 2]. Purification of crude hordothionin by ion-exchange chromatography resulted in two different fractions, designated HTH-1 and HTH-2, which most likely resemble α- and ß-hordothionin. *In vitro* growth inhibition experiments in solid and liquid media, indicated that these hordothionins, and the purothionins from wheat endosperm, were toxic to *C. michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *sepedonicus* and *X. campestris* pv. *vesicatoria* at the micromolar level. Thionins were not toxic to *Erwinia* and *Pseudomonas* spp., which is in contrast to the results obtained for purothionin by Fernandez de Caleya *et al.* [1972], who reported that *P. solanacearum* was one of the most sensitive bacteria. This could be the result of differences in strains or media used for testing, although we used a number of different strains and similar medium. The *in vitro* toxicity of hordothionins to *Clavibacter* and *Xanthomonas* spp. suggested that hordothionin encoding genes might be potential candidates for engineering bacterial disease resistance into solanaceous crops, especially tomato and potato. Since no cloned hordothionin encoding sequences were available to us, we decided to chemically synthesize such genes making use of the many advantages of synthetic genes [chapter 3]. Synthetic and semi-synthetic genes were constructed coding for different hordothionin precursor proteins, aimed at studying expression of the mature hordothionin in cytosol and secretion into the apoplast. To study their performance, all genes were expressed in tobacco. Analysis of transgenic plants revealed that no hordothionin was produced in plants containing gene constructs without a signalpeptide [chapter 4]. In contrast, plants harboring gene constructs containing a signalpeptide, the mature hordothionin and an acidic peptide coding sequence, which in structure resemble the natural gene from barley, accumulated high amounts of the mature protein. In plants harboring gene constructs without an acidic peptide, accumulation of the mature hordothionin was reduced more than tenfold. None of the plant cells secreted the hordothionins into the

apoplast of leaves. In addition, protoplasts derived from transgenic plants did not secrete hordothionins into the medium, which indicated that the targeting signal for intracellular retention must be located within the mature protein domain itself. The acidic peptide most likely facilitates transport through the membranes and does not determine, or at least not solely, the ultimate localization of the mature hordothionin. In most proteins initially made as prepropeptides, the targeting information is retained in the pro-part of the precursor and deletion of the pro-part encoding sequence often results in secretion into the apoplast [reviewed in Chrispeels, 1991]. This research shows that this is not true for the hordothionin precursor peptide. Partially purified extracts from leaves of these transgenic tobacco plants were toxic to *C. michiganensis* subsp. *michiganensis*, when tested *in vitro*, indicating that hordothionin is produced in its biologically active form. As expected, transgenic tobacco plants exhibiting high hordothionin expression levels were not resistant to *P. syringae* pv. *tabaci*, which is restricted to the intercellular spaces. On the other hand, these results are contradictory to those reported by Carmona *et al.* [1993], who reported growth inhibition and reduced symptom development after inoculation of transgenic tobacco plants, exhibiting high hordothionin expression levels, with another strain of this bacterium. These results are difficult to reconcile with the intracellular localization of the mature, antibacterial hordothionin. Nevertheless, the reported reduced growth of *P. syringae* pv. *tabaci* in transgenic plants [Carmona *et al.*, 1993], which was only measured for two days after inoculation, most likely has no significant value under field conditions. In addition to the results obtained with transgenic tobacco plants, tomato plants containing the construct performing best in tobacco were not resistant to *C. michiganensis* subsp. *michiganensis* [chapter 5], which invades the vascular system. Growth of *X. campestris* pv. *vesicatoria*, which multiplies in the intercellular spaces, was also not affected in these plants. However, when spray-inoculated with a bacterial suspension of the latter, these transgenic tomato plants showed much lesser symptoms compared to control plants. These findings await further testing under field conditions favorable for this pathogen to spread and infect tomato plants.

In summary, the results described in the first part of this thesis indicate that biologically active hordothionins can be expressed up to high levels in transgenic plants, depending on the construct used for transformation. However, hordothionins are not secreted by the plant cell. Therefore, hordothionins cannot be used to control growth of bacterial phytopathogens that are restricted to the apoplast or vascular tissue. Hordothionins might however be applicable for engineering resistance to those bacteria that infect plants via wounds or degrade plant cell walls, thus resulting in a close contact between the antibacterial hordothionin and the bacterium. Resistance of transgenic plants to rot-causing bacteria that degrade cell walls was not tested yet since these bacteria were not sensitive *in vitro* to hordothionins. This will however be tested in the near future.

7.3 Application of cecropin B for engineering resistance

Since hordothionins were not toxic to *Erwinia* spp. *in vitro* [chapter 2], which cause serious decrease of crop yield in a number of plants species, the feasibility of cecropin B for engineering bacterial disease resistance was examined. Cecropin B originates from an insect, the giant silkmoth (*Hyalophora cecropia*), has a very broad toxicity spectrum and is much more toxic to bacteria compared to hordothionins when tested *in vitro* [Nordeen *et al.*, 1992]. A number of genes were constructed coding for different cecropin B precursor peptides, aimed at studying the expression in cytosol or secretion into the apoplast. Expression of these gene constructs in tobacco revealed that they were actively transcribed [chapter 6]. However, the peptide could never be detected. In fact, incubation of a synthetic cecropin B peptide with

plant cell extracts revealed that the protein is degraded rapidly. Boiling of extracts prior to incubation with the peptide, and incubation in the presence of chymostatin, and to a lesser extent in the presence of PMSF, suggested that the protein is degraded by plant endogenous proteases, most likely serine proteases. Moreover, transgenic tobacco plants exhibiting high cecropin B-specific mRNA levels were not resistant to *P. solanacearum* and *P. syringae* pv. *tabaci*, which are highly sensitive to cecropin B *in vitro* [Nordeen *et al.*, 1992]. The expression of SB37, a cecropin B-analogue that differs only few amino acids compared to insect cecropin B, in tobacco, also did not result in resistance to *P. solanacearum* [Jaynes et al., 1993]. Moreover, the peptide could also not be detected in transgenic plants. However, in transgenic tobacco plants containing another cecropin B-analogue encoding sequence, namely for Shiva-1 that differs in approximately half of the amino acids, extremely low amounts of the peptide could be detected. Analysis of these transgenic plants indicated that they were resistant to *P. solanacearum* [Jaynes *et al.*, 1993].

In summary, the absence of observable levels of cecropin B peptide in transgenic tobacco plants and moreover, the absence of resistance of transgenic plants expressing high levels of cecropin B-mRNA, suggest that a successful application of (unmodified) cecropin B genes for engineering resistance into solanaceous crops, is highly unlikely. However, exact knowledge of the type of protease that degrades cecropin B in tobacco plant cell extracts, in conjunction with the reported toxicity of numerous cecropin B-analogues that have been made, might facilitate the construction of specific cecropin B-analogues that are not degraded by this protease by altering the primary amino acid sequence (protein engineering). Nevertheless, we must keep in mind that the modification may not interfere with toxicity of the peptide for the pathogen under study, and hence that only a limited number of changes can be made. These results also suggests that it is worthwhile to study the feasibility of using cecropin B encoding sequences for engineering resistance into other crops that do not produce such proteases. This is currently under investigation in a new project.

7.4 Other possibilities for engineering resistance to bacteria

Some other approaches using genetic engineering technology which may prove effective are:

1. The introduction of plant genes that confer disease resistance, into the genome of a susceptible host. Recently the *Pto* gene from tomato, conferring resistance to *P. syringae* pv. *tomato*, was cloned [Martin *et al.*, 1993]. The introduction of such genes into susceptible hosts however has the same disadvantages mentioned above for the traditional introgression of resistance traits.
2. The introduction and expression of genes interfering with some essential step in pathogenesis for which the pathogen most likely has not yet evolved an overcoming strategy. A number of potential genes could be derived from the pathogen itself ("parasite-derived resistance") and might be broadly applicable in engineering resistance [Sanford and Johnston, 1985]. Key products from the parasite, if present in excess or at the wrong developmental stage in the plant, could inhibit infection and/or pathogenesis without affecting the host. The first genetically engineered plants which showed resistance to bacterial infection applying this strategy were reported in 1989. Expression of a tabtoxin-resistant acetyltransferase, isolated from the bacterial phytopathogen *P. syringae* pv. *tabaci*, in tobacco plants conferred resistance to infection [Anzai *et al.*, 1989]. More recently, expression of a toxin-resistant target enzyme, isolated from *P. syringae* pv. *phaseolicola*, in sensitive plants resulted in insensitivity towards the toxin and resistance to bacterial infection [De la Fuente-Martínez *et al.*, 1992]. Expression of detoxifying or toxin-resistant target enzymes in susceptible plants to engineer

resistance is only applicable for bacteria producing toxins or related substances and will only be effective against these specific bacteria.

3. The introduction and pathogen-induced expression of an avirulence gene of which the primary product induces a hypersensitive response on appropriate cultivars containing the complementary resistance gene. An example of such a gene is the *avr4* gene from the fungus *Cladosporium fulvum* [Joosten *et al.*, 1994].
4. The introduction and expression of proteins that are lytic for bacterial cell walls. Recently, expression of bacteriophage T4 lysozyme in potato has been shown to result in significant lower tissue maceration after infection of tubers with *E. carotovora* subsp. *atroseptica* [Düring *et al.*, 1993].

7.5 Other applications of hordothionins

Recently, experiments performed by several groups indicated that thionins exhibit a strong fungicidal effect *in vitro* for a large number of plant pathogenic fungi [Cammue *et al.*, 1992; Terras *et al.*, 1993]. These results, and recent findings that phospholipase A2, which is activated in cultured mouse fibroblast cells and human erythrocytes upon treatment with thionins [Angerhofer *et al.*, 1990; Osorio e Castro *et al.*, 1989], might be involved in resistance of potato to the fungus *Phytophthora infestans* [Kawakita *et al.*, 1993], suggest that it is worthwhile to test transgenic plants for enhanced resistance to fungi. This awaits further testing of transgenic plants.

7.6 References

Angerhofer CK, Shier WT, Vernon LP: Phospholipase activation in the cytotoxic mechanism of thionin purified from nuts of *Pyrularia pubera*. Toxicon 28: 547-557 (1990).

Anzai H, Yoneyama K, Yamaguchi I: Transgenic tobacco resistant to a bacterial disease by the detoxification of a pathogenic toxin. Mol Gen Genet 219: 492-494 (1989).

Cammue BPA, De Bolle MFC, Terras FRG, Proost P, Van Damme J, Rees SB, Vanderleyden J, Broekaert WF: Isolation and characterization of a novel class of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. J Biol Chem 267: 2228-2233 (1992).

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. The Plant Journal 3: 457-462 (1993).

Chrispeels MJ: Sorting of proteins in the secretory system. Annu Rev Plant Physiol Plant Mol Biol 42: 21-53 (1991).

De la Fuente-Martínez JM, Mosqueda-Cano G, Alvarez-Morales A, Herrera-Estrella L: Expression of a bacterial phaseolotoxin-resistant ornithyl transcarbamylase in transgenic tobacco confers resistance to *Pseudomonas syringae* pv. *phaseolicola*. Bio/Technology 10: 905-909 (1992).

Düring K, Porsch P, Fladung M, Lörz H: Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. Plant J 3: 587-598 (1993).

Elphinstone JG: Soft rot and blackleg of potato. Techn Info Bull 21. International Potato Center (CIP), Lima, Peru (1987).

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).

Fox RTV, Manners JG, Myers A: Ultrastructure of tissue disintegration and host reactions in potato tubers infected by *Erwinia carotovora* var. *atroseptica*. Potato Res 15: 130-145 (1972).

Goto M: Fundamentals of bacterial plant pathology. Academic Press, Inc., San Diego (1992).

Jaynes JM, Nagpala P, Destéfano-Beltrán L, Huang JH, Kim JH, Denny T, Cetiner S: Expression of a cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum*. Plant Sci 89: 43-53 (1993).

Joosten MHAJ, Cozijnsen TJ, De Wit PJGM: Host resistance to a fungal tomato pathogen lost by a single base-

pair change in an avirulence gene. Nature 367: 384-386 (1994).
Kawakita K, Senda K, Doke N: Factors, affecting in vitro activation of potato phospholipase A2. Pl Sci 92: 183-190 (1993).
Kelman A: The bacterial wilt caused by *Pseudomonas solanacearum*. North Carolina Agric Exp Station Techn Bull 99 (1953).
Martin C, French ER: Bacterial wilt of potato. Techn Info Bull 13. International Potato Center (CIP), Lima, Peru (1985).
Martin GB, Brommonschenkel SH, Chunwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED, Tanksley SD: Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262: 1432-1436 (1993).
Nordeen RO, Sinden SL, Jaynes JM, Owens LD: Activity of cecropin SB37 against protoplasts from several plant species and their bacterial pathogens. Plant Sci 82: 101-107 (1992).
Osorio e Castro VR, Van Kuiken BA, Vernon LP: Action of a thionin isolated from nuts of *Pyrularia pubera* on human erythrocytes. Toxicon 27: 501-510 (1989).
Sanford JC, Johnston SA: The concept of parasite-derived resistance- deriving resistance genes from the parasite's own genome. J Theor Biol 113: 395-405 (1985).
Stall RE, Cook AA: Multiplication of *Xanthomonas vesicatoria* and lesion development in resistant and susceptible pepper. Phytopathol 56: 1152-1154 (1966).
Stefani E: *In situ* detection of *Clavibacter michiganensis* ssp. *sepedonicus* in potato stems using the fluorescent antibody technique. Phytopathol medit 28: 53-56 (1989).
Stewart W: Isolation and proof of structure of wildfire toxin. Nature 229: 174-178 (1971).
Terras FRG, Schoofs HME, Thevissen K, Osborn RW, Vanderleyden J, Cammue BPA, Broekaert WF: Synergistic enhancement of the antifungal activity of wheat and barley thionins by radish and oilseed rape 2S albumins and by barley trypsin inhibitors. Plant Physiol 103: 1311-1319 (1993).
Wallis FM: Ultrastructural histopathology of tomato plants infected with *Corynebacterium michiganense*. Physiol Plant Pathol 11: 333-342 (1977).

SUMMARY

Bacterial diseases can cause a drastic decrease of yield in certain crops. Breeding for bacterial disease resistance therefore is of utmost necessity. Up to now, traditional plant breeding was the only method to reach this goal. Recent developments in genetic engineering technology however provide novel ways for the production of disease resistant plants. This thesis describes the results of two research projects that have been undertaken to investigate the potential of such a novel way, namely the introduction and expression of genes coding for antibacterial proteins in plants. In the first project, the potential of the hordothionins from barley (*Hordeum vulgare*) endosperm has been investigated, and in the second, the potential of cecropin B from the giant silkmoth (*Hyalophora cecropia*).

In the first part of chapter 1, the literature available on plant thionins is presented. General information on the different thionin types, homology, occurrence in nature, molecular structure and toxicity for microorganisms and cultured cells is listed. Since their role in nature has not yet been established, although a putative role in plant defense has been proposed by several groups, special emphasis is put on the numerous divergent activities displayed by the different thionins in conjunction with possible modes of action and biological roles. Data collected from literature indicate that thionins might expose their toxic activity *in vitro* by several mechanisms: by acting as a thiol intermediate in reducing and oxidizing proteins or by direct binding to DNA and/or RNA or by interaction with the phospholipid membrane, acting most likely on Ca^{2+}-channels or -pumps, and/or Ca^{2+}-ATPases. In the second part of chapter 1, an overview of the literature on insect cecropins is presented. General information on molecular structure, toxicity and known mode of action of these proteins is presented. Special emphasis is put on one of them, cecropin B, which was under investigation in the second research project.

Our first choice was to investigate the feasibility of using thionin encoding sequences for engineering bacterial disease resistance into solanaceous crops. The hordothionins originating from barley endosperm were chosen because of the availability of nucleotide and amino acid sequences. To establish the potential of hordothionins, the toxicity for plant pathogenic bacteria had to be determined first. To this end the thionins from wheat and barley endosperm were isolated (chapter 2). The thionins were purified in few steps from flour by petroleum-ether extraction and hydrochloric acid treatment of the resulting lipoprotein, followed by ion-exchange chromatography. The hordothionins were separated into two forms, HTH-1 and HTH-2, probably reflecting the two forms described in literature, namely α- and ß-hordothionin. *In vitro* experiments indicated that purothionin, hordothionin and HTH-1 and HTH-2 were equally toxic to *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker on tomato, *C. michiganensis* subsp. *sepedonicus*, the causal agent of ring rot on potato and *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of a foliage and fruit spot disease on tomato and pepper. *Erwinia* spp. and *Pseudomonas* spp. were insensitive.

Since hordothionins appeared to be toxic for a number of bacteria that affect tomato and potato, several hordothionin encoding constructs were made (chapter 3). Analysis of published hordothionin cDNA clones indicated that the 5 kD mature hordothionin was made as a much larger precursor protein. This precursor consisted of an amino-terminal signalpeptide, followed by the mature hordothionin exhibiting antibacterial activity, and a carboxy-terminal so-called acidic peptide of unknown function. Since no such cDNA or genomic hordothionin clones were available to us, we decided to chemically synthesize the genes making use of the many advantages of synthetic genes. In chapter 3, the design and construction of seven different α- and ß-hordothionin encoding constructs is described. Gene

constructs were made to study expression in cytosol and secretion into the apoplast. Genes were designed for optimal expression in solanaceous crops by adapting the codon usage and optimizing the translation initiation region, and for convenience in subsequent cloning steps.

The seven hordothionin gene constructs made were cloned in a plant expression vector under the control of the constitutive cauliflower mosaic virus 35S promoter and introduced in tobacco to study their expression, processing of precursors, sorting and biological activity (chapter 4). Analysis of a large number of transgenic plants indicated that the signalpeptide was essential for expression, whereas the acidic peptide facilitated transport of mature hordothionin and increased accumulation at least tenfold compared to plants harboring constructs without this acidic peptide coding sequence. Fractionation of protoplasts prepared from transgenic plants indicated that hordothionin accumulated in the microsomes and membranes and was not secreted into the medium. In addition, hordothionin was not secreted into the apoplast in intact leaves. The hordothionin partially purified from leaves of transgenic tobacco plants exhibited *in vitro* toxicity for *C. michiganensis* subsp. *michiganensis*, at comparable doses as the hordothionin from barley endosperm. The tobacco phytopathogen *P. syringae* pv. *tabaci* was however not affected in growth in transgenic tobacco plants exhibiting high hordothionin expression levels (chapter 4).

In vitro growth inhibition experiments indicated clearly that hordothionin was toxic for a few bacteria that were pathogenic on tomato and potato. For this reason, the best performing construct was introduced in tomato (chapter 5). Transgenic plants were selected exhibiting high hordothionin expression levels, selfed to obtain plants homozygous for the transgene and evaluated for resistance. No differences were observed between control plants and transgenic tomato plants exhibiting high hordothionin expression levels upon infection with *C. michiganensis* subsp. *michganensis*. In addition, no growth inhibition of *X. campestris* pv. *vesicatoria* was observed in leaves of these transgenic plants upon infiltration of a bacterial suspension. However, less symptoms were visible on these transgenic plants upon spray-inoculation with a suspension of the latter.

Since hordothionins were not toxic to *Erwinia* spp. and *Pseudomonas* spp. *in vitro* (chapter 2), a second antibacterial protein was chosen exhibiting toxicity to these bacteria. Three different cecropin B gene constructs were made and introduced in tobacco. Analysis of transgenic plants indicated that the genes were transcribed into mRNA, however the protein could not be detected (chapter 6). By mixing a synthetic cecropin B peptide with different tobacco cell extracts, it appeared that the peptide was rapidly degraded, whereas boiling of the extracts prior to mixing did not result in cecropin B degradation, suggesting protease degradation. This was confirmed by experiments which indicated that the protease inhibitors chymostastin and to a lesser extent PMSF, also inhibited degradation. Transgenic plants exhibiting high cecropin B-mRNA levels were nevertheless evaluated for resistance to two pathogens, *P. solanacearum* and *P. syringae* pv. *tabaci*. Results from these experiments clearly indicated that transgenic plants were not resistant.

Finally, in chapter 7 a general discussion on the topic described in this thesis is presented. Also, other potential approaches to obtain bacterial disease resistant plants are discussed and other applications of hordothionin encoding sequences.

SAMENVATTING

Bacterieziekten kunnen een drastische reductie in gewasopbrengst tot gevolg hebben. Het is daarom zeer belangrijk dat er plantenveredelingsonderzoek, gericht op bacterieresistentie, wordt verricht. Tot nu toe kon dat alleen via de klassieke kruisingsveredeling. Recente ontwikkelingen in de plantenbiotechnologie bieden echter een aantal nieuwe mogelijkheden. In dit proefschrift zijn de resultaten van een tweetal projecten samengevat die tot doel hadden om de mogelijkheden van zo'n nieuwe strategie, namelijk de expressie van antibacteriële eiwitten in planten, te onderzoeken. In het kader van het eerste project werd de mogelijke toepassing van de hordothioninen uit het endosperm van gerst (*Hordeum vulgare*) onderzocht, en in het tweede project, de mogelijke toepassing van cecropine B uit de zijdemot (*Hyalophora cecropia*).

In het eerste deel van hoofdstuk 1 is de literatuur aangaande de thioninen uit planten samengevat. Er wordt algemene informatie gegeven over de verschillende typen thioninen, hun overeenkomsten en voorkomen in de natuur, hun moleculaire structuur en toxiciteit voor microorganismen en cellen in cultuur. Ondanks het feit dat er wordt verondersteld dat thioninen een rol in de afweer van planten tegen microorganismen hebben, is de exacte biologische rol van thioninen niet geheel duidelijk. Om deze reden wordt er in dit hoofdstuk speciale aandacht besteed aan de zeer uiteenlopende aktiviteiten die thioninen vertonen, samen met mogelijke werkingsmechanismen en een mogelijke biologische rol. Literatuur gegevens tonen aan dat thioninen hun toxisch effect zouden kunnen teweegbrengen via verschillende mechanismen, namelijk door als thiol-intermediair eiwitten te reduceren en te oxideren, door directe binding aan DNA en/of RNA, of door interactie met de phospholipide membraan, zodanig dat er een effect is op Ca^{2+}-kanalen en pompen, en/of Ca^{2+}-ATPases. In het tweede deel van hoofdstuk 1 wordt een overzicht gegeven van de literatuur aangaande andere antibacteriële eiwitten, namelijk de cecropinen uit insecten. Er wordt aandacht besteed aan de moleculaire structuur, de toxiciteit en het bekende werkingsmechanisme van deze eiwitten. Speciale aandacht wordt gewijd aan cecropine B, waarvan een mogelijke toepassing werd onderzocht in het kader van het tweede project.

Onze eerste keuze betrof de mogelijke toepassing van thionine coderende sequenties voor het verkrijgen van bacterieresistentie. Er werd gekozen voor de hordothioninen uit het endosperm van gerst vanwege de beschikbaarheid van nucleotide- en aminozuur sequenties. Om hun eventuele toepasbaarheid te onderzoeken moest allereerst de toxiciteit voor plant pathogene bacteriën worden bepaald. Daartoe werden de thioninen uit het endosperm van tarwe en gerst geisoleerd (hoofdstuk 2), middels een petroleum-ether extractie van bloem, gevolgd door incubatie van het eiwit-lipide complex met zoutzuur en ionenwisselaarchromatografie. De hordothioninen konden gescheiden worden in twee vormen, HTH-1 en HTH-2, welke naar alle waarschijnlijkheid identiek zijn aan α- en ß-hordothionine. *In vitro* groeiremmingsexperimenten toonden aan dat zowel purothionine, hordothionine als HTH-1 en HTH-2 dezelfde toxiciteit vertoonden. Deze peptiden waren toxisch voor *Clavibacter michiganensis* subsp. *michiganensis*, de veroorzaker van een verwelkingsziekte in tomaat, *C. michiganensis* subsp. *sepedonicus*, de veroorzaker van ringrot in aardappel en *Xanthomonas campestris* pv. *vesicatoria*, de veroorzaker van een blad- en vruchtvlekkenziekte in tomaat en paprika. *Erwinia* spp. en *Pseudomonas* spp. waren echter ongevoelig.

Vanwege hun toxiciteit voor een aantal bacteriën welke tomaat en aardappel aantasten, werden er enkele hordothionine coderende genconstructen gemaakt (hoofdstuk 3). Analyse van gepubliceerde hordothionine cDNA sequenties toonden aan dat het 5 kD rijpe hordothionine aanvankelijk wordt gemaakt als een veel groter precursor eiwit. Dit bestaat uit een amino-terminaal signaalpeptide, gevolgd door het antibacteriële rijpe eiwit en een

zogenaamd zuur-polypeptide met onbekende functie. Aangezien wij niet de beschikking hadden over hordothionine cDNA en/of genomische klonen, werd besloten deze genen chemisch te synthetiseren, waarbij gebruik werd gemaakt van de vele voordelen die synthetische genen bieden. In hoofdstuk 3 wordt het ontwerp en de constructie van een zevental verschillende α- en ß-hordothionine coderende genconstructen beschreven. Er werden constructen gemaakt ter bestudering van hordothionine expressie in het cytoplasma en secretie naar de apoplast. De genen werden geoptimaliseerd voor expressie in *Solanaceae* door aanpassing van het codongebruik en de translatie initiatie regio, en voor handigheid ten behoeve van toekomstige kloneringen.

Deze zeven hordothionine genconstructen werden voor expressie in planten gekloneerd achter de constitutieve cauliflower mosaic virus 35S promoter en geintroduceerd in tabak om expressie, processing van precursors, lokalisering en biologische aktiviteit te onderzoeken (hoofdstuk 4). Analyse van een groot aantal transgene tabaksplanten toonde aan dat het signaalpeptide essentieel is voor expressie. De zure-polypeptide coderende sequentie is dat niet, maar vergemakkelijkt wel het transport van het rijpe eiwit en verhoogt de accumulatie van het rijpe eiwit minstens tienmaal. Fractionering van protoplasten, welke waren geisoleerd van bladeren van transgene tabaksplanten, toonden aan dat hordothionine zich ophoopt in de microsomen en membranen, en niet gesecreteerd wordt naar het medium. Er vindt eveneens geen secretie naar de apoplast plaats in bladeren van deze transgenen. Het gedeeltelijk opgezuiverde hordothionine uit bladeren van transgene tabaksplanten vertoonde wel *in vitro* toxiciteit voor *C. michiganensis* subsp. *michiganensis* bij vergelijkbare concentraties als het hordothionine uit het endosperm van gerst. De groei van *P. syringae* pv. *tabaci* werd echter niet geremd in deze transgene planten (hoofdstuk 4).

Hordothionine vertoonde *in vitro* toxiciteit voor bacteriën die pathogeen zijn voor tomaat en aardappel (hoofdstuk 2). Om deze reden werd het construct dat het beste tot expressie kwam in tabak, eveneens geintroduceerd in tomaat (hoofdstuk 5). Transgene tomatenplanten werden geselecteerd welke hoge hordothionine expressie vertoonden. Deze werden zelfbevrucht ter verkrijging van homozygote planten, die werden onderzocht op resistentie. Transgene tomateplanten bleken niet resistent te zijn tegen *C. michiganensis* subsp. *michiganensis*. Ook de groei van *X. campestris* pv. *vesicatoria* werd niet geremd in bladeren van deze planten na infiltratie. Daarentegen vertoonden transgene tomateplanten met hoge hordothionine expressie minder symptomen na inoculatie met *X. campestris* pv. *vesicatoria* door verneveling op blad.

Aangezien hordothionine geen *in vitro* toxiciteit vertoonde voor *Pseudomonas* spp. en *Erwinia* spp. (hoofdstuk 2), werd gekozen voor een tweede antibacterieel peptide dat wel toxisch is voor deze bacteriën. Er werden drie verschillende cecropine B coderende genconstructen gemaakt, welke geintroduceerd werden in tabak (hoofdstuk 6). Analyse van transgene tabaksplanten toonde aan dat de genen overgeschreven werden in mRNA. Het eiwit kon echter niet aangetoond worden. Nader onderzoek leerde dat cecropine B peptide zeer snel afgebroken werd na mengen met tabaksextracten. Koken van de extracten voor toevoeging van het peptide resulteerde daarentegen niet in afbraak, wat er op duidt dat het cecropine B mogelijk wordt afgebroken door proteases. Dit werd bevestigd door experimenten waaruit bleek dat de protease inhibitor chymostatine, en in mindere mate PMSF, de afbraak remden. Toetsing van de transgene tabaksplanten met hoge cecropine B-mRNA nivo's op bacteriëresistentie toonde onomstotelijk aan dat deze niet resistent waren tegen *P. syringae* pv. *tabaci* en *P. solanacearum*.

Tenslotte wordt er in hoofdstuk 7 een algemene discussie over dit onderzoek gepresenteerd. Daarnaast worden er in dit hoofdstuk andere mogelijkheden om via biotechnologie te komen tot bacterieresistente planten opgesomd, alsook andere toepassingen van hordothionine coderende sequenties gegeven.

Account

Most of the results presented in this thesis have been published before, or will be published in the near future. The content of this thesis has been based on the following publications:

Florack D.E.A., L. Visser, L. van Vloten-Doting, F. Heidekamp & W.J. Stiekema, 1990. Synthetic hordothionin genes as tools for bacterial disease resistance breeding. In: J.J. Dekkers, H.C. van der Plas, D.H. Vuijck, (Eds.). Agricultural biotechnology in focus in the Netherlands. Pudoc, Wageningen, the Netherlands, pp. 39-46.

Florack D.E.A., B. Visser, Ph.M. de Vries, J.W.L. van Vuurde & W.J. Stiekema, 1993. Analysis of the toxicity of purothionins and hordothionins for plant pathogenic bacteria. Neth. J. Plant Pathol. 99: 259-268.

Stiekema W.J., B. Visser & D.E.A. Florack, 1993. Is durable resistance against viruses and bacteria attainable via biotechnology? In: Th. Jacobs, J.E. Parlevliet, (Eds.). Durability of disease resistance. Kluwer Academic Publishers, the Netherlands, pp. 71-81.

Florack D.E.A., W.G. Dirkse, B. Visser, F. Heidekamp & W.J. Stiekema, 1994. 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. Plant Mol. Biol. 24: 83-96.

Florack D., Allefs S., Bollen R., Bosch D., Visser B. & W.J. Stiekema, 1994. Expression of giant silkmoth cecropin B genes in tobacco. Transgenic Res.: in press.

Florack D.E.A. & W.J. Stiekema, 1994. Thionins: properties, possible biological roles and mechanisms of action. Plant Mol. Biol.: in press.

Curriculum vitae

Dion Florack werd geboren op 23 april 1963 te Maastricht. Hij behaalde het diploma VWO (Gymnasium-ß) in 1981 aan het Stedelijk Lyceum & Havo te Maastricht. In dat jaar begon hij de studie Plantenveredeling aan de toenmalige Landbouwhogeschool te Wageningen. Deze studie werd afgesloten in januari 1988 met het behalen van het Doctoraalexamen Plantenveredeling met als hoofdvakken Moleculaire Biologie en Plantenveredeling, en als bijvak Tuinbouwplantenteelt. In januari 1988 werd hij aangesteld als wetenschappelijk onderzoeker bij de Stichting Ital, later het DLO Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO), alwaar het in dit proefschrift beschreven onderzoek is uitgevoerd als onderdeel van twee projecten. Het eerste project werd gefinancierd door de Programma Commissie Landbouwbiotechnologie (PcLB) en werd uitgevoerd van 1 januari 1988 tot 31 mei 1990. Dit project had als titel: "Protection of *Solanaceae* against phytopathogenic microorganisms by the introduction of synthetic hordothionin genes". Het tweede project werd gefinancierd door PBTS, en werd uitgevoerd van 1 juni 1990 tot 31 mei 1993. Dit project had als titel: "Protection of willow against the watermark disease by the expression of antibacterial proteins". Vanaf 1 oktober 1993 is hij tijdelijk werkzaam bij het CPRO-DLO in een project dat er op gericht is om via biotechnologie het vaasleven van snijrozen te verbeteren.

NAWOORD

Bij deze wil ik gaarne allen bedanken die direct of indirect, hebben bijgedragen aan de totstandkoming van dit proefschrift. Sedert het begin van dit onderzoek in januari 1988 zijn dat er velen geweest. Allereerst Freek Heidekamp die op het idee kwam om synthetische hordothionine genen te maken en te toetsen op hun bruikbaarheid in de gentechnologie. Wim Dirkse voor zijn assistentie bij het opzetten van al het werk destijds en het geouwehoer aan de labtafel. Lous van Vloten-Doting en mijn promotor Ab van Kammen, die vanaf het allereerste begin kritische gesprekspartners waren. Dit geldt ook voor Andy Pereira met wie ik jarenlang dezelfde kamer deelde. Gerard Bredemeijer en Henk Burg wil ik bedanken voor hun hulp bij het zuiveren van de thioninen uit gerst. Jim van Vuurde en Ineke de Vries voor hun hulp bij het testen van de toxiciteit van thioninen voor phytopathogene bacteriën. Menno Drost, Peter Stad, Dick Vermeer en Frank Klinge voor fotografie. Pim Lindhout en Gerard Pet voor het testen van de resistentie van transgene tomaat voor *Clavibacter michiganensis* subsp. *michiganensis*. Jeffrey Jones for his advice and assistance with testing tomato plants for resistance to *Xanthomonas campestris* pv. *vesicatoria*. Ellie Munsterman voor het bepalen van de groei van *Pseudomonas syringae* pv. *tabaci* in transgene tabak en de vele boerewijsheden op de labvloer. Erik Dullaard, Peter de Jong, Dick Geurtsen, Johan Hulsman en Hans Jansen voor hun zorg voor de vele honderden transgenen. Sjefke Allefs voor zijn hulp bij de toetsen met *Pseudomonas solanacearum*. De "studenten" Brigit Kusters, Rik Bollen en Annette Valkering voor hun bijdragen. Willem Geutjes en Egbert van Kranen voor hun technische assistentie, vooral wat betreft het "nat" houden van de PC-II klimaatcel. Mijn familie die altijd klaar stond als het nodig was. Het thuisfront, Marieke, Marlou, Dion jr. en Floran, die er voor zorgden dat ik niet alleen met mijn werk bezig was. En last-but-not-least Bert Visser, Dirk Bosch en Willem Stiekema voor het dagelijks begeleiden van het werk door de jaren heen en hun kritiek op teksten, artikelen en experimenten.

Tevens wil ik hen, die ik niet met name heb genoemd, bedanken voor hun bijdragen in welke vorm dan ook. In het bijzonder diegenen die op dit moment, en in de nabije toekomst, nog een aantal experimenten zullen uitvoeren met de transgene tabaks- en tomateplanten. Deze zullen nog uitvoerig getest worden op veldresistentie tegen *X. campestris* pv. *vesicatoria*. Dit laatste betreft een veldproef in Florida in samenwerking met Jeffrey Jones van de Universiteit van Florida, voor het eerst uit te voeren in het najaar van 1994. Daarnaast zullen deze planten getoetst worden op resistentie tegen schimmels.

Dion Florack.