Journal of Biochemistry and Molecular Biology, Vol. 34, No. 3, May 2001, pp. 201-205

JOURNAL OF
Biochemistry
and
Molecular Biology

Molecular Cloning and Characterization of a Flower-specific Thionin in Chinese Cabbage

Bae Gyo Jung, Yeon Ok Choi, Kyun Oh Lee, Yong Hun Chi, Soon Suk Kang, Seung Sik Lee, Soo Kwon Park, Jung Ro Lee, Chae Oh Lim and Sang Yeol Lee*

Graduate School of Applied Life Sciences, Gyeongsang National University, Chinju 660-701, Korea

Received 2 December 2000, Accepted 26 January 2001

Thionins are a family of low molecular weight cysteine-rich antimicrobial peptides. We isolated a cDNA encoding thionin gene from a flower bud cDNA library of Chinese cabbage (*CFT*). The gene contains 611 bp nucleotides with 60 bp, and 150 bp untranslated regions at its N- and C-terminal, respectively. The deduced amino acid sequence encoded 133 amino acids containing precursor polypeptide. The protein reveals that the precursor has a tripartite structure: a putative signal sequence at the N-terminus, followed by a mature thionin peptide, and a C-terminal acidic domain, which facilitates transport of the mature thionin through membrane. Genomic Southern blot analysis suggests that the CFT gene may be present as a single or two copy gene in the Chinese cabbage genome. Northern blot analysis shows that the gene is specifically expressed in flowers, but not in leaves, stems, or roots. When we analyzed the antifungal activity of the recombinant CFT protein, which was expressed in *E. coli* using the truncated cDNA region corresponding to the mature protein part, it was not active on fungal growth inhibition.

Keywords: Antifungal protein, Gene cloning, Flower-specific expression, Thionin.

Introduction

Plants have evolved a variety of different mechanisms to cope with the constant threat of pathogenic attacks (Lee *et al.*, 1999). They developed physical barriers and antimicrobial substances such as phytoalexins, PR-proteins and thionins (Epple *et al.*, 1995; Thevissen *et al.*, 1999). Thionins are a class of basic and cysteine-rich, low molecular weight proteins found in a wide range of plant species, and they play an important role in the plant defense system (Florack *et al.*, 1994; Orru *et al.*, 1997). Based on the number of amino acid and disulfide bonds present in the mature protein, thionin proteins have been classified into the a, b, and g types (Colilla *et al.*, 1990). Although the biological function of thionin has not yet been established, members of this group have been shown to be toxic to bacterial and fungal phyotopathogens (Reuber *et al.*, 1998), plant protoplasts and animal cells (Garcia-Olmedo *et al.*, 1989; Reimann-Philipp *et al.*, 1989). Thionins have also been shown to inhibit DNA, RNA, and protein synthesis, and to act as secondary thiol messengers (Garcia-Olmedo *et al.*, 1989). The antimicrobial properties of thionins make them potential candidates for engineering transgenic plants against pathogenic bacteria or fungi. Recently, it was reported that the overexpression of endogenous thionin in transgenic *arabidopsis* resulted in an increased resistance to *Fusarium oxysporum* f sp matthiolae (Epple *et al.*, 1997). Thionins from the leaves, or the endosperm of barley, were shown to be toxic to plant pathogenic fungi and bacteria (Bohlmann *et al.*, 1998; Vossen *et al.*, 2000; Molina *et al.*, 1993). The accumulation of barley leaf thionin is induced by phytopathogenic fungi, such as *Septoria nodorum* and *Drechslera graminea* (Titarenko *et al.*, 1993; Vale *et al.*, 1994). Thionin in *A. thaliana* was induced by wounding, or inoculation with *F. oxysporum* f sp matthiolae (Vignuteli *et al.*,1998; Thevissen *et al.*, 2000).

In order to broaden our knowledge of thionin properties, this paper describes the molecular cloning and characterization of cDNA encoding a thionin (*CFT*), whose mRNA is specifically expressed in flower tissues of Chinese cabbage.

Materials and Methods

Materials For Northern and Western blot analyses, Chinese cabbage (*Brassica campestris* L. ssp. pekinensis) was grown in a

The sequence data reported in this paper has been submitted to GenBank under the accession number of AF090836.

*To whom correspondence should be addressed.
Tel: 82-55-751-5958; Fax: 82-55-759-9363
E-mail: sylee@nongae.gsnu.ac.kr

growth chamber with a 16/8 h light/dark cycle, a temperature of 20°C, and a relative humidity of 70%.

Cloning of a cDNA encoding thionin from Chinese cabbage A phage-mid flower bud cDNA library of Chinese cabbage was screened using a partial clone of thionin cDNA obtained from a random sequencing project as a probe. From among six positive clones, the longest *Eco*RI/*Xho*I cDNA insert was sequenced by the dideoxy sequencing method using a Taq dye primer in an automated DNA sequencer (Applied Biosystems Model 373A) (Lee *et al.*, 1999).

Southern and Northern blot analysis Genomic DNA was prepared from leaves (Dellaporta *et al.*, 1983). Southern blot hybridization was carried out under high stringency conditions. For Northern blot analysis, various tissues of Chinese cabbage were divided into sections, and total RNAs were extracted as described previously (Cheong *et al.*, 1999; Choi *et al.*, 1999).

Expression of CFT protein in *E. coli* For expression of CFT in *E. coli*, the mature form of the *CFT* gene was cloned into the *pGEX2T* expression vector (Lee and Kim, 1999). The resulting construct, *pGEX-CFT*, was introduced into *E. coli* BL21(DE3)pLysS and grown in luria-broth medium containing 100 μg/ml ampicillin. When the absorbance of the culture at 600 nm had reached 0.6-0.8, isopropyl-β-D-thiogalactosylpyranoside (IPTG) was added to a final concentration of 0.4 mM. After an additional 3 h incubation, the cells were collected by centrifugation, frozen in liquid nitrogen, and stored at -70°C until use. The glutathione S-transferase fusion protein was isolated from the cytosolic fraction of the *E. coli* by glutathione agarose affinity gel filtration. Afterwards the N-terminal glutathione S-transferase portion of the protein was removed with thrombin, and the native form of the CFT was used for the analysis of their biochemical properties.

Results and Discussion

Molecular cloning of a cDNA encoding thionin from Chinese cabbage To isolate tissue-specifically regulated new genes from flower buds of Chinese cabbage, we prepared a phage-mid flower bud cDNA library, and carried out random sequencing of flower bud-specific cDNA clones (Lim *et al.*, 1996). This led to the identification of a partial cDNA clone Expressed Sequence Tag (EST)-F1644, which closely matched the thionin gene in *Arabidopsis thaliana*. Using the [^{32}P]-labeled *EST-F1644* clone as a probe, a full length cDNA encoding the protein was isolated from the same cDNA library, named *CFT*, which represents the Chinese cabbage flower-specific thionin. The nucleotide and deduced amino acid sequence of *CFT* is shown in Fig. 1. The nucleotide sequence of the clone will appear under the accession number of AF090836 in the GenBank nucleotide sequence database. The CFT protein contains 133 amino acids, which include the N-terminal signal sequence, followed by a 46 amino acids mature thionin and a C-terminal acidic domain. This result

```
GAATTCGGATCCCTCTCTCTCTACGTACATTTTGTGACCAAACAAGTAATCAGTCAAAAG   60
ATGGAAGGCAAAACTGTGATTTTAGGTGTGATCATAATGAGTCTTGTCATGGCGCAGAAT  120
 M  E  G  K  T  V  I  L  G  V  I  I  M  S  L  V  M  A  Q  N    (20)
CAAGTCGAAGCAAAGATTTGTTGCCCCAGAACCATTGACAGAAACATATATAATGCATGC  180
 Q  V  E  A▼K  I  C  C  P  R  T  I  D  R  N  I  Y  N  A  C    (40)
CGTCTTACTGGAGCCTCTATGACAAATTGTGCAAACCTCAGTGGATGCAAAATCGTTTCT  240
 R  L  T  G  A  S  M  T  N  C  A  N  L  S  G  C  K  I  V  S    (60)
GGGACGACATGTCCTCCGGGATATACACATGACATTCTCCAAAACTATGGTGATGCTGTC  300
 G  T  T  C  P  P  G  Y  T  H▼D  I  L  Q  N  Y  G  D  A  V    (80)
AATGAATACTGCAAGTTGGGGTGTGCATCCTCTGTGTGCGGTGCCTTAACCACTCTCAAG  360
 N  E  Y  C  K  L  G  C  A  S  S  V  C  G  A  L  T  T  L  K   (100)
AACTCGATGCAAGTAAATTGTGAAGGAGCGGTTTCACAATGCACCAACGCATGTTCTAAT  420
 N  S  M  Q  V  N  C  E  G  A  V  S  Q  C  T  N  A  C  S  N   (120)
TTCTGCACCAAGGGCTCAGCTAAAGTAGTTGAAACTGCCTAACAAGCATATCCGCTATGT  480
 F  C  T  K  G  S  A  K  V  V  E  T  A  *                     (113)
ATTAATTGTATGTGTTGTTGTTTTACATGTTTTCAATAATTGTCGTGTATGTGACAAATT  540
GCTAGACTCTTTTGCTATTTCCAAGTTCTATGTGACGTAGAAGGTCATCGATCCCTAAAA  600
AAAAAAAAAA                                                    611
```

Fig. 1. Nucleotide and deduced amino acid sequences of the *CFT* cDNA. The nucleotide sequence of CFT is numbered starting with the first nucleotide of the insert. Amino acid numbering starts with the first methionine residue. Bold letters indicate the sequences of putative mature thionin. The vertical arrows denote the probable cleavage sites.

```
                                          ▼
CFT          MEG----KTVILGVIIMSLVMAQNQVEAKICCPRTIDRNIYNACRLTGAS    46
Crambin      MEG----KTVILSVLIMSLVMSYNQVEATTCCPSIVARSNFNVCRLPGTS    46
ATH-Thi2.2   MEG----KTVISSLLIMSLVLAQIQVEAKICCPTKDDRSVYFVCMLSVSS    45
ATH-Thi2.1   MKG----RILILSLLIMSLVMAQVQVEAKICCPSNQARNGYSVCRIRFS-    44
BLT          MATNKSIKSVVICVLILGLVLEQVQVEAKSCCKNTTGRNCYNACRFAGGS    50
DB4          MAPSKSIKSVVICVLILGLVLEQVQVEGKSCCKATLARNCYNTCHFAGGS    50
HTH1         MG----LKGVMVCLLILGLVLEQVQVEGKSCCRSTLGRNCYNLCRVRGAQ    46
             *     . ..  ..*..**..  * *.. **    .*. .  *    .

CFT          MTNCAN-LSGCKIVSGTTCPPGYTH/DILQNY--GDAVNEYCKLGCASSV    92
Crambin      EAICAT-YTGCIIIPGATCPGDYAN/NILKNSAQGNAVNEYCKWGCASSV    94
ATH-Thi2.2   QFYCLL-KSKCKNTSQTICPPGYTN/DILENS--GDAVNEYCKLGCASSV    91
ATH-Thi2.1   KGRCNQ-VSGCQM--SDTCPRGWVN/AILENSA--DATNEHCKLGCETSV    88
BLT          RPVCATA-CGCKIISGPTCPRDYPK/LNLLPESGEPNATEYCTIGCRTSV    98
DB4          RPVCAGA-CRCKIISGPKCPSDYPK/LNLLPESGEPDVTQYCTIGCRNSV    98
HTH1         K-LCAG-VCRCKLTSSGKCPTGFPK/LALVSNSDEPDTVKYCNLGCRASN    94
                *     *      ** .. .  *       .  ..*. ** .*

CFT          CGALTTLKNSMQVNC-EGAVSQCTNACSNFCTKG---SAKVVETA  133
Crambin      CGALTNLQNSDAREIVNGAVRQCTNACSDFCTNG---SAKAVETA  136(64.7%)
ATH-Thi2.2   CGALTTLQNEDASKVLSEAVEQCTKACSSVCTGG---STAAVKSA  133(62.4%)
ATH-Thi2.1   CGAMNTLQNSDASEIVNGISEQCAKGCSIFCTKSYVVPPGPPKLL  133(47.45)
BLT          CDNM---DNVSRGQEMKFDMGLCSNACARFCNDGEVIQSVEA---  137(42.1%)
DB4          CDNM---DNVFRGQEMKFDMGLCSNACARFCNDGAVIQSVEA---  137(38.3%)
HTH1         CDYM--VNAAADDEEMKLYLENCGDACVNFCNGDAGLTSLTA---  134(31.6%)
             *. .   ..    . .    *...*  *. .    .
```

Fig. 2. Comparison of deduced amino acid sequence of CFT with other thionins. The deduced amino acid sequences of CFT (this work) is aligned with crambin of *C. abyssinica* (Epple *et al.*, 1998), ATH-Thi2.1, ATH-Thi2.2 of *A. thaliana* (Epple *et al.*, 1995), BLT of barley (Gausing, 1987), DB4 of *Hordeum vulgare* (Bohlmann *et al.*, 1987), and HTH1 of *H. vulgare* (Rodriguez-Palenzuela *et al.*, 1988). Asterisks (*) denote positions perfectly conserved, and dots (.) mark well-conserved positions. Gaps are introduced to maximize the alignment. The amino acid sequence identity of the proteins shared with CFT is shown in the last column (%). An arrow indicates the N-terminal signal peptide cleavage site, and the slash (/) denotes the cleavage site of the C-terminal propeptide. The alignment was computed with the CLUSTAL program of the PCGENE software package (Genofit SA, Geneva, Switzerland/Intelligenetics, Mountain View, CA).

indicates that CFT is synthesized as a putative preproprotein. It may be processed by at least two steps of co-translational removal, both for the N-terminal signal peptide and for the C-

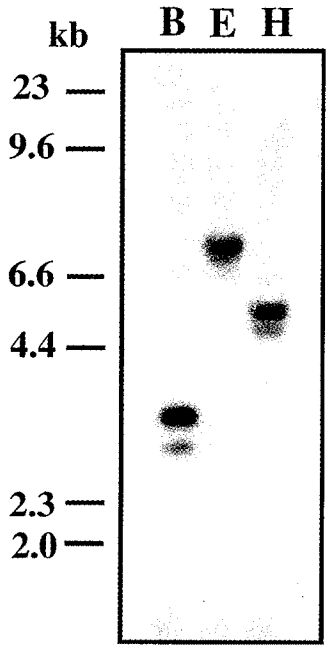

Fig. 3. Genomic Southern blot analysis of the *CFT* gene. Purified genomic DNA digested with *Bam*HI (B), *Eco*RI (E), or *Hin*dIII (H) respectively was size-fractionated on a 0.8% agarose gel. The blot was hybridized with the [^{32}P]-labeled full-length CFT cDNA. Size markers in kb are shown on the left.

terminal acidic domain. Database searches of the predicted CFT amino acid sequence revealed significant sequence similarity to the thionins isolated from *arabidopsis*, barley, *Crambe abyssinica* (Epple *et al.*, 1995). Fig. 2 represents the alignment of CFT with other thionin proteins in plant sources. It shows the highest sequence identity to crambin (64.7%) in *C. abyssinica* and ATH-Thi2.2 (62.4%) in *A. thaliana* (Epple *et al.*, 1995). When we introduced gaps to maximize the fit of the amino acid sequences, six cysteines and one tyrosine were perfectly conserved in CFT with the same amino acid intervals. This suggests that the residues may play an important role in its function.

Genomic complexity of the *CFT* gene in Chinese cabbage We examined the genomic complexity of the *CFT* gene in the Chinese cabbage genome by Southern blot analysis using the full length *CFT* cDNA as a probe. When genomic DNA (digested with *Bam*HI, *Eco*RI or *Hin*dIII, restriction enzymes that did not cleave within the coding sequence) was hybridized under high stringency conditions, one or two restriction fragments were hybridized with the probe (Fig. 3.) The result of a Southern blot analysis showed that the *CFT* gene consisted of a single, or two copies of a gene family in the Chinese cabbage genome.

Flower-specific expression of the *CFT* gene To identify the possible physiological functions of the *CFT*, we examined its gene expression in terms of RNA levels in various tissues

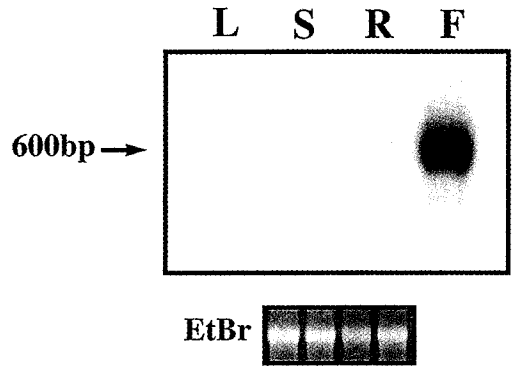

Fig. 4. Tissue-specific expression of the *CFT* in different tissues of Chinese cabbage. Total RNAs from the leaves (L), stems (S), roots (R) or flowers (F) were isolated and subjected to Northern blot analysis. The mRNA detected by the radio-labeled probe was about 0.6kb in size. For equal loading of RNA in each lane, the gel was examined after staining with EtBr.

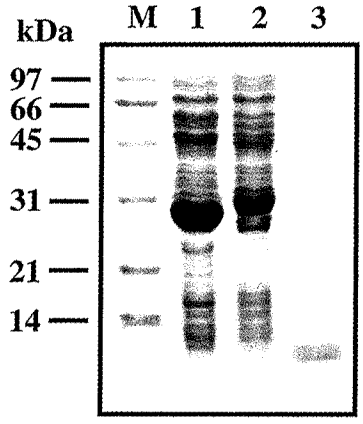

Fig. 5. Expression of the *CFT* gene in *E. coli*. Expression of the glutathione S-transferase fusion protein was induced by adding 0.4 mM IPTG. The culture was continued at 30°C for 4 hr. Fusion proteins obtained from the cytosolic fraction of transformed cells were separated on 15% SDS-PAGE before (lane 1), or after (lane 2) IPTG induction. After purification of the fusion protein, the N-terminal portion of the glutathione S-transferase protein was removed by thrombin, and the purified ΔCFT protein was loaded in lane 3. M indicates the molecular size marker.

by Northern blot analysis under high stringency hybridization conditions. We used total RNA prepared from leaf, stem, root, and flower tissues of Chinese cabbage. In this experiment, the *CFT* gene clearly hybridized to a single transcript approximately 0.6kb in size, and revealed a tissue-specific expression pattern. The *CFT* mRNA was specifically expressed in the flowers of Chinese cabbage, but not in the leaves, stems, or roots (Fig. 4). The conclusion from the results is that the *CFT* protein may have an important role in plant flowers as a defense protein against the various pathogenic microorganisms, or as an essential protein for other functions of a flower differentiation or its development.

Expression of the CFT in *E. coli* We expressed the truncated form of *CFT* (ΔCFT) cDNA corresponding to the mature protein by using the *E. coli pGEX2T* expression vector to test the functional activity of the CFT protein. After the resulting DNA construct (*ΔCFT-pGEX2T*) was transformed into *E. coli* BL21(DE3), the protein was over-expressed by 0.4 mM IPTG. The majority of the recombinant ΔCFT protein was found in the soluble fraction, which facilitated the purification by affinity chromatography. After purification of the recombinant protein by means of a glutathione agarose column, the N-terminal portion of glutathione S-transferase was removed from the fusion protein by thrombin (Fig. 5). The molecular weight of the over-expressed fusion-protein was estimated to be 31kDal, and the mature protein that cleavaged the N-terminal glutathione S-transferase protein was 5kDal on a 15% SDS-PAGE gel. The protein is well-matched with the molecular weight expected from the calculated molecular size of the cloned cDNA insert. Using the purified ΔCFT protein, we tested the antifungal activity of the protein for several fungi such as *Fusarium oxysporium*, *Trichoderma reesei*, and *Neurospora crassa*. The ΔCFT protein (in protein concentrations ranging from 10 to 100 ug, loaded onto the PDA agar plates containing actively growing fungal hyphae) showed no inhibitory effect on tested fungi (data not shown), even though the N-terminal amino acid sequence of the expressed ΔCFT protein determined by Edman degradation was accurately matched with the N-terminal 12 amino acid sequences deduced from the corresponding core cDNA sequence (Fig. 1). The reason why the bacterially expressed ΔCFT protein, containing the mature protein part, showed no antifungal activity may be due to the incorrect protein modification at Gly 44 (putative site of N-myristoylation) and Asn 52 (putative site of N-glycosylation), or to protein miss-folding. Therefore, in future, we need to express an active CFT protein by using other eukaryotic expression systems in order to examine the biochemical properties.

Acknowledgments This research was supported in part by the G7 project (#; 02-08-06) and in part by the NRL program (project #; 2000-N-NL-01-C-236) supported by MOST, Korea.

References

Bohlmann, H. and Apel, K. (1987) 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.* **201**, 446-454.

Bohlmann, H., Vignutelli, A., Hilpert, B., Miersch, O., Wasternack, C. and Apel, K. (1998) Wounding and chemicals induce expression of the *Arabidopsis thaliana* gene Thi2.1, encoding a fungal defense thionin, via the octadecanoid pathway. *FEBS Lett.* 437, 281-6.

Cheong, N. E., Choi, Y. O., Lee, K. O., Kim, W. Y., Jung, B. G., Chi, Y. H., Jeong, J. S., Kim, K., Cho, M. J. and Lee, S. Y. (1999) Molecular cloning, expression, and functional characterization of a 2Cys-peroxiredoxin in Chinese cabbage. *Plant Mol. Biol.* **40**, 825-834.

Choi, Y. O., Cheong, N. E., Lee, K. O., Jung, B. G., Hong, C. H., Jeong, J. H., Chi, Y. H., Kim, K., Cho, M. J., and Lee, S. Y. (1999) Cloning and expression of a new isotype of the peroxiredoxin gene of Chinese cabbage and its comparison to 2Cys-peroxiredoxin isolated from the same plant. *Biochem. Biophys. Res. Commun.* **258**, 768-771.

Colilla, F. J., Rocher, A. and Mendez, E. (1990) Gamma-Purothionins: amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm. *FEBS Lett.* **270**, 191-194.

Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) A plant mini-preparation: VersionII. *Plant Mol. Biol. Rep.* **1**, 19-22.

Epple, P., Apel, K. and Bohlmann, H. (1995) An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. *Plant Physiol.* **109**, 813-820.

Epple, P., Apel, K. and Bohlmann, H. (1997) Overexpression of an endogenous thionin enhances resistance of Arabidopsis against *Fusarium oxysporum*. *Plant Cell* **9**, 509-520.

Epple, P., Vignutelli, A., Apel, K. and Bohlmann, H. (1998) Differential induction of the *Arabidopsis thaliana* Thi2.1 gene by *Fusarium oxysporum* f. sp. matthiolae. *Mol. Plant Microbe Interact.* **11**, 523-529.

Florack, D. E. A. and Stiekema, W. J. (1994) Thionins: properties, possible biological roles and mechanisms of action. *Plant Mol. Biol.* **26**, 25-37.

Garcia-Olmedo, F., Rodriguez-Pelenzuela, P., Hernandez-Lucas, C., Ponz, F., Marana, C., Carmona, M. J., Lopez-Fando, J., Fernandez, J. A. and Carbonero, P. (1989) The thionins: a protein family that includes purothonins, viscotoxins and crambins. Oxford Surv. *Plant Mol. Cell Biol.* **6**, 31-60.

Gausing, K. (1987) Thionin genes specifically expressed in barley leaves. *Planta* **171**, 241-247.

Lee, D. and Kim, D-H. (1999) Heterologous expression of lignin peroxidase H2 in *Escherichia coli*: *In vitro* refolding and activation. *J. Biochem. Mol. Biol.* **32**, 486-491.

Lee, H. S., Choi, S. Y., and Kwon, O-S. (1999) Isolation and characterization of cDNA encoding pyridoxal kinase from ovine liver. *J. Biochem. Mol. Biol.* **32**, 502-505.

Lee, K. O., Kim, M. G., Jang, H. H., Lee, J. Y., Kim, S.C. and Lee, S.Y. (1999) Biochemical properties of a chitin-binding class III chitinase in pumpkin leaves. *J. Biochem. Mol. Biol.* **32**, 541-546.

Lim, C. O., Kim, M. G., Hwang, I. and Cho, M. J. (1996) Expressed sequence tags of Chinese cabbage flower bud cDNA. *Plant Physiol.* **111**, 577-588.

Molina, A., Ahl-Goy, P., Fraile, A., Sanchez-Monge, R. and Gracia-Olmedo, F. (1993) Inhibition of bacterial and fungal plant pathogenes by thionins of types I and II. *Plant Sci.* **92**, 169-177.

Oita, S., Ohnishi-Kameyama, M. and Nagata, T. (2000) Binding of barley and wheat alpha-thionins to polysaccharides. *Biosci Biotechnol. Biochem.* **64**, 958-64.

Orru, S., Scaloni, A., Giannattasio, M., Urech, K., Pucci, P. and Schaller, G. (1997) Amino acid sequence, S-S bridge arrangement and distribution in plant tissues of thionins from Viscum album. *Biol. Chem.* **378**, 989-96.

Reimann-Philipp, U., Schrader, G., Martinoia, E., Barkholt, V. and Apel, K. (1989) Intracellular thionins of barley. A second group of leaf thionins closely related to but distinct from cell wall-bound thionins. *J. Biol. Chem.* **264**, 8978-84.

Reuber, T. L., Plotnikova, J. M., Dewdney, J., Rogers, E. E., Wood, W. and Ausubel, F. M. (1998) Correlation of defense gene induction defects with powdery mildew susceptibility in Arabidopsis enhanced disease susceptibility mutants. *Plant J.* **16**, 473-85.

Rodriguez-Palenzuela, P., Pintor-Toro, J. A., Carbonero, P. and Gracia-Olmedo, F. (1988) Nucleotide sequence and endosperm-specific expression of the structural gene for the toxin α-hordothionin in barley (*Hordeum vulgare L.*). *Gene* **70**, 271-281.

Thevissen, K., Terras, F. R. and Broekaert, W. F. (1999) Permeabilization of fungal membranes by plant defensins inhibits fungal growth. *Appl. Environ. Microbiol.* **65**, 5451-5458.

Thevissen, K., Cammue, B. P., Lemairem, K., Winderickx, J., Dickson, R. C., Lester, R. L., Ferket, K. K., Van Even, F., Parret, A. H., Broekaert, W. F. (2000) A gene encoding a sphingolipid biosynthesis enzyme determines the sensitivity of *Saccharomyces cerevisiae* to an antifungal plant defensin from dahlia (*Dahlia merckii*). *Proc Natl. Acad. Sci. USA* **97**, 9531-9536.

Titarenko, E., Hargreaves, J., Keon, J. and Gurr, D. J. (1993) In *Mechanisms of Plant Defense*, Fritig, B. and Legrand, M. (eds.), pp. 308-311. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Vale, G. P., Torrigiani, E., Gatti, A., Delogu, G., Porta-Puglia, A., Vannacci, G. and Cattivelli, L. (1994) Activation of genes in barley roots in response to infection by two *Drechslera graminea* isolates. *Physiol. Mol. Plant Pathol.* **44**, 207-215.

Van Der Vossen, E. A., Van Der Voort, J. N., Kanyuka, K., Bendahmane, A., Sandbrink, H., Baulcombe, D. C., Bakker, J., Stiekema, W. J. and Klein-Lankhorst, R. M. (2000) Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. *Plant J.* **23**, 567-76.

Vignuteii, A., Wasternack, C., Apel, K. and Bohlmann, H. (1998) Systemic and local induction of an *Arabidopsis* thionin gene by wounding and pathogens. *Plant J.* **14**, 285-295.