Expression of the α-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens

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Summary

Thionins are cysteine-rich, 5 kDa polypeptides which are toxic to plant pathogens *in vitro*. Expression of the gene encoding α-thionin from barley endosperm, under the 35S promoter from cauliflower mosaic virus, conferred to transgenic tobacco enhanced resistance to the bacterial plant pathogens *Pseudomonas syringae* pv. tabaci 153 and *P. syringae* pv. syringae. The barley α-thionin gene, which has two introns, was correctly spliced in tobacco. The α-thionin in transgenic plants had the expected mobility in the gradient, when separated by high-performance liquid chromatography, reacted with monospecific antibodies and showed the expected antibiotic properties *in vitro*.

Introduction

The achievement of enhanced resistance to pathogens through the transgenic expression of genes encoding possible defence proteins is of interest both to test the plausibility of the proposed defence role and as a necessary step to manipulate an important agronomic trait. In a recent review (Bowles, 1990), defence-related proteins have been grouped into three classes, namely those that affect the extracellular matrix and therefore have a passive role in defence, those that appear in relation to a defence response but whose function is unknown, and those that act directly as deterrents. Included in the latter class are inhibitors of fungal and bacterial plant pathogens, such as chitinase and (1–3)-β-glucanase, endohydrolases that have been found to limit fungal growth by degrading major components of the fungal cell wall (see Boller, 1988), ribosome-inactivating proteins (RIP), which disrupt protein synthesis (Leah *et al.*, 1991), thaumatin-like proteins (Hejgaard *et al.*, 1991), the zeamatin family (Vigers *et al.*, 1991) and the thionins (see García-Olmedo *et al.*, 1989). Many of the genes encoding these proteins are expressed both under developmental regulation and as part of a defence response. Plant storage tissues generally contain considerable amounts of proteins that are either toxic or inhibitory towards pathogens and predators, which suggests that they may have a defence function (García-Olmedo *et al.*, 1987). In cereal endosperm, a substantial fraction of the dry matter content is represented by several types of such proteins (García-Olmedo *et al.*, 1987, 1989), one of which is the thionin family (García-Olmedo *et al.*, 1989). Thionins are cysteine-rich polypeptides of about 5 kDa that are also present in cereal leaves (Bohlmann and Apel, 1987; García-Olmedo *et al.*, 1989; Gausing, 1987; Reimann-Philipp *et al.*, 1989) and whose toxicity to plant pathogens *in vitro* has been previously established (Fernandez de Caleya *et al.*, 1972). They are synthesized as precursors in which the amino acid sequence of the mature protein is preceded by a typical signal peptide and followed by an acidic C-terminal sequence (Hernández-Lucas *et al.*, 1986; Ponz *et al.*, 1983; Rodriguez-Palenzuela *et al.*, 1988). The corresponding genes have two introns that interrupt the nucleotide sequence coding for the acidic peptide (Bohlmann *et al.*, 1988; Rodriguez-Palenzuela *et al.*, 1988).

Recently, plants expressing transgenes encoding chitinase (Broglie *et al.*, 1991; Jach *et al.*, 1992) and RIP (Logemann *et al.*, 1992) have shown increased protection against fungal pathogens. We now report that the transgenic expression of barley α-thionin in tobacco confers enhanced resistance to bacterial pathogens.

Results

Expression of thionin gene constructions in tobacco

Thionin chimeric genes were constructed by cloning appropriate fragments from a wheat α1-thionin cDNA and from the barley α-thionin genomic DNA into a derivative of plasmid pMPK110 (Eckes *et al.*, 1986) between the CaMV-35S promoter and the 3′-region of the octopine synthase gene (Figure 1a). Tobacco plants were transformed with both gene constructions and kanamycin-resistant regenerants were checked for nopaline synthesis and then for thionin mRNA (data not shown). The phenotype of the transformed plants was undistinguishable from that of the wild-type throughout their development. Seeds from these plants were germinated in the presence of kanamycin and 3:1 segregation of resistance to this antibiotic was observed in most progenies. After selection, the plants were grown in sterile soil and leaves from each progeny were pooled. Total RNA was extracted and steady-state levels of thionin mRNA relative to that in barley endosperm

were determined by a dot-blot procedure (Figure 1b). The size of the thionin mRNA corresponding to the two gene constructions, as estimated by Northern analysis (Figure 1c), was about the same, which suggested that the two introns in the barley α-thionin gene (Rodriguez-Palenzuela *et al.*, 1988) had been properly spliced. This was confirmed by amplification of the sscDNA from tobacco leaves by the thermostable polymerase chain reaction (PCR), using primers which flanked the region that included the two introns (Figure 1d). The DNA amplified from leaves corresponding to the barley α-thionin gene construction was of identical size to that amplified from a barley α-thionin cDNA clone, and its nucleotide sequence was that expected for a correct splicing of the gene. This is in contrast with the inefficient splicing of introns of some other genes from monocots when expressed in dicots (Keith and Chua, 1986).

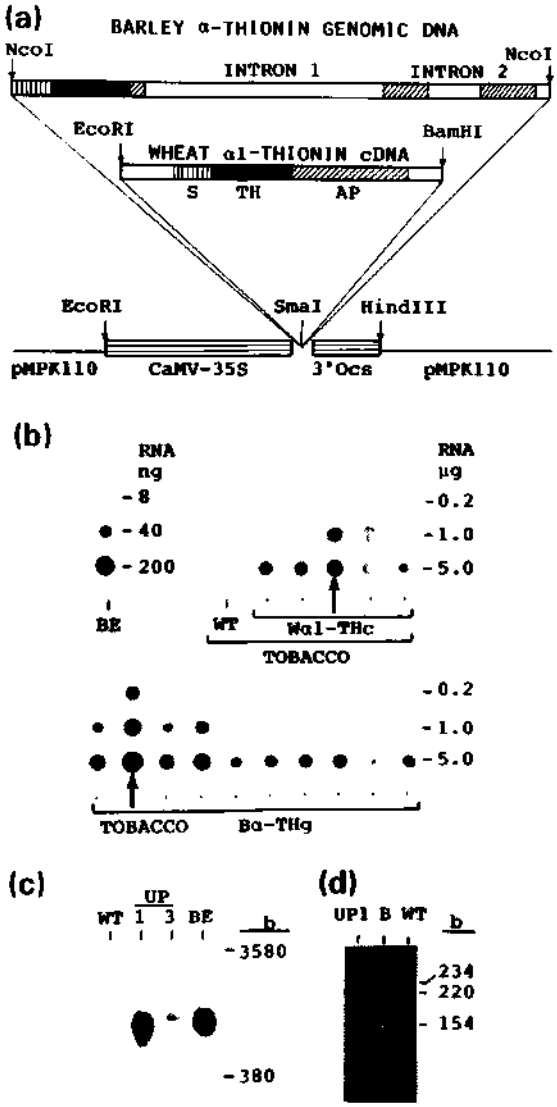

Figure 1. Expression of barley and wheat thionin genes under the CaMV-35S promoter in transgenic tobacco.

(a) Gene fusions with barley α-thionin genomic DNA and with an α1-thionin cDNA from wheat, designated Bα–THg and Wα1–THc, respectively. Regions corresponding to the signal peptide (S), the mature thionin (TH) and the C-terminal acidic peptide (AP) are indicated.

(b) Dot-blot analysis of thionin mRNA steady-state levels in pooled leaves from the progenies of the Bα–THg and Wα1–THc transformants, from non-transformed tobacco (WT) and from barley endosperm (BE). Cloned barley α-thionin cDNA (Ponz *et al.*, 1986) was used as probe. Total RNA was blotted in all cases. Arrows indicate the progenies selected for Northern-blot analysis (UP1, Bα–THg; UP3, Wα1–THc).

(c) Northern-blot analysis of total RNA from transgenic tobacco leaves (UP1 and UP3) and from barley endosperm (BE); 15 µg of RNA per lane for tobacco and 1 µg per lane for barley were applied. The same probe as above was used.

(d) Amplification by the polymerase chain reaction (PCR) of the region flanked by the sequences of primers TTGGCCCTTGTGTCAAACTC and ATTGACACAAGCATCACCAC in the barley α-thionin gene. In lane B is the amplification product from an α-thionin cDNA clone (Ponz *et al.*, 1986).

Analysis of thionins in transgenic tobacco plants

Low levels of protein (<1 µg g^{-1} fresh weight) were detected in the leaves of plants transformed with the wheat cDNA construction (Wα1–THc) using a dot-blot filter assay in which crude extracts were reacted with monospecific antibody and ^{35}S-protein A (data not shown). Higher levels of reactive protein were detected in the leaves of plants carrying the barley α-thionin gene (Bα–THg) by the same assay, and the correct processing of the thionin precursor was investigated in the progeny of the transformant with the highest thionin mRNA level (UP1) by subjecting protein extracts to high resolution reverse-phase high-performance liquid chromatography (Figure 2a). The fraction eluting exactly as the mature barley α-thionin (TH fraction; Figure 2a) was collected from this sample and from protein extracts of non-transformed tobacco leaves with and without purified α-thionin added at the homogenization stage as internal standard. Equivalent aliquots of the TH fraction from the three extracts were dot-blotted and reacted with α-thionin monospecific antibodies (Figure 2b). The signal obtained with the immunodetection reagent for the TH fraction from the transformed plant corresponded to an estimated α-thionin concentration of 20 µg per g of fresh leaves (4×10^{-6} mol kg^{-1}). The potato bacterial pathogen *Clavibacter michiganensis* subsp. *sepedonicus*, routinely used to test thionins *in vitro* (minimum inhibitory concentration = 2×10^{-6} M), was inhibited by an aliquot of the TH fraction from the transformed plant, as expected from its estimated α-thionin content (Figure 2c).

Protection tests in vivo

Protection tests *in vivo* were carried out with the pathogen, *Pseudomonas syringae* pv. tabaci 153, a sensitive strain for which α-thionin minimum inhibitory concentration *in vitro* was 3×10^{-6} M. Transgenic plants from the progenies of transformants UP1, UP2, UP6 and UP7, which had the highest expression levels among those carrying the

in response to both biotic and abiotic stress (Bohlman *et al.*, 1988; Fisher *et al.*, 1989) is also in line with a defence role. The *in vivo* protection effect reported here lends further support to the hypothesis.

Increased ability of tobacco seedlings to survive in soil infested with the fungal pathogen *Rhizoctenia solani* has been achieved through the transgenic expression of chitinases both from bacteria (Jach *et al.*, 1992) and from plants (Broglie *et al.*, 1991), and similar results have been also obtained by expressing RIP (Logemann *et al.*, 1992). *In vitro* tests carried out with a wide range of bacterial and fungal pathogens (Fernandez de Caleya *et al.*, 1972 and unpublished data) indicate that genetic variants of different thionin types have similar activity for a given pathogen and that fungal pathogens tend to be less sensitive than bacterial ones. Nevertheless, many of them should be also susceptible of control *in vivo* by attainable expression levels of thionin genes in transgenic plants.

An increasing number of genes encoding antimicrobial peptides are being characterized at present not only from plants, as already indicated, but from other organisms, including animals (see Boman and Hultmark, 1987; Lehrer *et al.*, 1991). The prospect of introducing all these genes, or combinations of them, into plants opens new avenues to engineer resistant plants which merit further exploration.

Experimental procedures

Gene constructions and plant transformation

The DNA fragments indicated in Figure 1a were released from the plasmid vectors with appropriate restriction enzymes: the *Nco*I/*Nco*I fragment from a genomic DNA clone corresponding to the α-thionin gene from barley (Rodriguez-Palenzuela *et al.*, 1988) and the *Eco*RI/*Bam*HI fragment from a cDNA clone encoding wheat α1-thionin (Maraña, unpublished data). These fragments were made blunt by treatment with Klenow's fragment of the *E. coli* DNA polymerase and cloned into the *Sma*I site of a derivative of plasmid pMPK110 (Eckes *et al.*, 1986). These constructions were inserted into a T1 plasmid derivative in *Agrobacterium tumefaciens* C58C1 (pGV3850 Km^R) by mobilization and co-integration (Eckes *et al.*, 1986; Jones *et al.*, 1985; Van Haute *et al.*, 1983). Transgenic plants of *Nicotiana tabacum* cv. Samsun NN were obtained by the leaf-disc infection procedure (Horsch *et al.*, 1985). Standard methods were used for nopaline determination (Otten and Schilperoort, 1978), RNA extraction, dot-blot and Northern-blot experiments, (^{32}P)-radioactive labelling of the cDNA probes by primer extension, and PCR amplification (Sambrook *et al.*, 1989; Verwoed *et al.*, 1989). The amplified fragments were directly sequenced by a published procedure (Bachman *et al.*, 1990).

Thionin analysis

Tobacco leaves were ground in liquid nitrogen and treated with 10%trichloroacetic acid in acetone at −20°C for 45 min, washed with cold acetone and air-dried (Damerval *et al.*, 1986). The acetone powder was extracted with 0.05 M H_2SO_4 (3 ml g^{-1} fresh weight) for 1 h and the extracted protein was precipitated by saturation with ammonium sulphate. After dialysis against water (Spectra/Por-7 membrane, Spectrum, Medican Ind. Inc., Houston, TX, USA), the precipitate was freeze-dried and either dissolved in water for direct quantitation of the crude extracts by a dot-blot procedure or dissolved in 0.1% trifluoroacetic acid, 2% acetic acid, and subjected to HPLC in a Beckman System Gold chromatograph, using an Ultrapor C-3 column (1 × 25 cm) and the indicated H_2O-acetonitrile gradient (0.1% trifluoroacetic acid). Both crude extracts and fractions collected from HPLC were quantitated by blotting into an Immobilon PVDF membrane (Millipore, Bedford, MA, USA) with the aid of a BioDot manifold (BioRad, Richmond, CA, USA), reaction with monospecific antibody and staining with the Protein-A Gold Kit from BioRad or with ^{35}S-Protein A (Amersham, UK) according to the manufacturer's instructions. The antibody was raised against barley α-thionin and purified by passage through an α-thionin–Sepharose column as described (Ponz *et al.*, 1983). The purified antibody was equally reactive with all known variants of endosperm thionins from barley and wheat (unpublished data). Purified α-thionin from barley was a gift from Dr. C. Hernández-Lucas.

Tests with pathogens

For *in vitro* tests with *Clavibacter michiganensis* subsp. *sepedonicus* (syn. *Corynebacterium sepedonicum*), bacteria (2×10^3 in 15 µl) were inoculated into microtiter wells to which 50 µl of nutrient broth (Oxoid, Basingstoke, UK) and 85 µl of the aliquot to be tested had been added. The amounts of thionin in the aliquot were determined immunochemically as above. Incubation was at 28°C for 28 h and growth was measured in a Titertek Multiskan Plus MKII (Flow Lab., Irvine, UK) at 492 nm.

For the *in vivo* tests with *Pseudomonas syringae* pv. tabaci 153, transformant and control plants were grown in sterile soil (200 g per plant) in multi-well plastic containers that were placed within unlidded plastic boxes (60 × 42 × 40 cm) and kept in a growth chamber (Heraeus, Balingen, Germany) at 75% relative humidity, 25°C, 16 h (day) and at 65% relative humidity, 22°C, 8 h (night). Prior to inoculation, relative humidity was raised to 90% (day) and 80% (night) for 24 h. Inoculation (15 µl of 10^5 cfu ml^{-1} in nutrient broth) was carried out by infiltration into the underside of intact leaves from 14-week-old plants at the three spots indicated by ink marks on the upper surface (Figure 3a). After inoculation, plants were kept at 80% relative humidity (day) and 75% (night). Inoculation of *P. syringae* pv. syringae was carried out at CIBA-GEIGY (Basel) by a single injection per leaf, at a lateral point of the midrib, with a bacterial suspension (10^7 cfu ml^{-1}).

Bacterial growth in infiltrated leaves was monitored by harvesting and homogenizing three leaves at each stage. Appropriate 10-fold dilutions were plated in the presence of rifampycin (100 µg ml^{-1}) and colonies counted. Data expressed as bacteria per cm^2 of leaf area.

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References

Bachman, B., Lüke, W. and Hunsmann, G. (1990) Improvement of PCR amplified DNA sequencing with the aid of detergents. *Nucl. Acids Res.* **18**, 1309.

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.* **207**, 446–454.

Bohlmann, H., Clausen, S., Behnke, S., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V. and Apel, K. (1988) 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.

Boller, T. (1988) Ethylene and the regulation of antifungal hydrolases in plants. *Oxf. Surv. Plant Mol. Cell Biol.* **5**, 145–174.

Boman, H.G. and Hultmark, D. (1987) Cell-free immunity in insects. *Ann. Rev. Microbiol.* **41**, 103–126.

Bowles, D.J. (1990) Defense-related proteins in higher plants. *Ann. Rev. Biochem.* **59**, 873–907.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J. and Broglie, R. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science*, **254**, 1194–1197.

Damerval, C., de Vienne, D., Zivy, M. and Thiellement, H. (1986) Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. *Electrophoresis*, **7**, 52–54.

Eckes, P., Rosahl, S., Schell, J. and Willmitzer, L. (1986) Isolation and characterization of a light-inducible, organ-specific gene from potato and analysis of its expression after tagging and transfer into tobacco and potato shoots. *Mol. Gen. Genet.* **205**, 14–22.

Fernandez de Caleya, R., Gonzalez, B., García-Olmedo, F. and Carbonero, P. (1972) Susceptibility of phytopathogenic bacteria to wheat purothionins *in vitro*. *Appl. Microbiol.* **23**, 998–1000.

Fisher, R., Behnke, S. and Apel, K. (1989) The effect of chemical stress on the intercellular fluid of barley leaves. *Planta*, **178**, 61–68.

García-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, L., Royo, J. and Carbonero, P. (1987) Plant proteinaceous inhibitors of proteinases and α-amylases. *Oxf. Surv. Plant Mol. Cell Biol.* **4**, 275–334.

García-Olmedo, F., Rodriguez-Palenzuela, P., Hernández-Lucas, C., Ponz, F., Maraña, C., Carmona, M.J., López-Fando, J., Fernández, J.A. and Carbonero, P. (1989) The thionins: A protein family that includes purothionins, viscotoxins and crambins. *Oxf. Surv. Plant Mol. Cell Biol.* **6**, 31–60.

Gausing, K. (1987) Thionin genes specifically expressed in barley leaves. *Planta*, **171**, 241–246.

Hejgaard, S., Jacobsen, S. and Svendsen, I. (1991) Two antifungal thaumatin-like proteins from barley grain. *FEBS Lett.* **291**, 127–131.

Hernández-Lucas, C., Royo, J., Paz-Ares, J., Ponz, F., García-Olmedo, F. and Carbonero, P. (1986) Polyadenylation site heterogeneity in mRNA encoding the precursor of the barley toxin β-hordothionin. *FEBS Lett.* **200**, 103–105.

Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) A simple and general method for transferring genes into plants. *Science*, **227**, 1229–1231.

Jach, G., Logemann, S., Wolf, G., Oppenheim, A., Chet, I., Schell, J. and Logemann, J. (1992) Expression of a bacterial chitinase leads to improved resistance of transgenic tobacco plants against fungal infection. *Biopractice*, **1**, 1–10.

Jones, J.D., Dunsmuir, P. and Bedbrook, J. (1985) High level expression of introduced chimaeric genes in regenerated transformed plants. *EMBO J.* **4**, 2411–2418.

Keith, B. and Chua, N.-H. (1986) Monocot and dicot pre-mRNAs are processed with different efficiencies in transgenic tobacco. *EMBO J.* **5**, 2419–2425.

Leah, R., Tommerup, H., Svendsen, I. and Mundy, J. (1991) Biochemical and molecular characterization of three anti-fungal proteins from barley seeds. *J. Biol. Chem.* **266**, 1564–1573.

Lehrer, R.I., Ganz, T. and Selsted, M.E. (1991) Defensins: endogenous antibiotic peptides of animals cells. *Cell*, **64**, 229–230.

Logemann, J., Jach, G., Tommerup, H., Mundy, J. and Schell, J. (1992) Expression of a barley ribosome-inactivating protein leads to increased fungal protection in transgenic tobacco plants. *Biotechnology*, **10**, 305–308.

Otten, L. and Schilperoort, R.A. (1978) A rapid microscale method for the detection of lysopine and nopaline dehydrogenase activities. *Biochem. Biophys. Acta*, **527**, 497–500.

Ponz, F., Paz-Ares, J., Hernández-Lucas, C., Carbonero, P. and García-Olmedo, F. (1983) Synthesis and processing of thionin precursors in developing endosperm from barley (*Hordeum vulgare* L.). *EMBO J.* **2**, 1035–1040.

Ponz, F., Paz-Ares, J., Hernández-Lucas, C., García-Olmedo, F. and Carbonero, P. (1986) Cloning and nucleotide sequence of a cDNA encoding the precursor of the barley toxin α-hordothionin. *Eur. J. Biochem.* **156**, 131–135.

Reimann-Philipp, U., Schrader, G., Martionia, E., Barkholt, V. and Apel, K. (1989) Intracellular thionins of barley. *J. Biol. Chem.* **264**, 8978–8984.

Rodriguez-Palenzuela, P., Pintor-Toro, J.A., Carbonero, P. and García-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.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Van Haute, E., Joos, H., Maes, M., Warren, G., Van Montagu, M. and Schell, J. (1983) Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for the reversed genetics of T1 plasmids of *Agrobacterium tumefaciens*. *EMBO J.* **2**, 411–418.

Verwoed, T.C., Dekker, B.M.M. and Hoekema, A. (1989) A small-scale procedure for the rapid isolation of plant RNAs. *Nucl. Acids Res.* **17**, 2362.

Vigers, A.J., Roberts, W.K. and Selitrennikoff, C.P. (1991) A new family of plant antifungal proteins. *Mol. Plant–Microbe Inter.* **4**, 315–323.