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 Boer, 1988), ribosome-inactivating proteins (RIP), which disrupt protein synthesis (Leah et al., 1991), thaumatin-like proteins (Heegaard 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; 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; Rodríguez-Palenzuela et al., 1988). The corresponding genes have two introns that interrupt the nucleotide sequence coding for the acidic peptide (Bohlmann et al., 1986; Rodríguez-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 α-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 ssDNA 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).

### 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 (Wa1–THc) using a dot-blot filter assay in which crude extracts were reacted with monospecific antibody and 35S-protein A (data not shown). Higher levels of reactive protein were detected in the leaves of plants carrying the barley α-thionin gene (Ba–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
Tobacco leaves were ground in liquid nitrogen and treated with cold acetone and air-dried (Damerval et al., 1986). The fragments were directly sequenced by a published procedure (Fernandez de Caleya 1972 and et al., 1992) further support to the hypothesis. Increased ability of tobacco seedlings to survive in soil infested with the fungal pathogen *Rhizoctonia 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 genes 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 *NcoI*/*NcoI* fragment from a genomic DNA clone corresponding to the α-thionin gene from barley (Rodriguez-Palenzuela et al., 1986) and the *EcoRI*/*BamHI* fragment from a cDNA clone encoding wheat α-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 *Smal* site of a derivative of plasmid pMPK110 (Eckes et al., 1988). These constructions were inserted into a T1 plasmid derivative in *Agrobacterium tumefaciens* C58C1 (pGV3850 Km*) by mobilization and co-integration (Eckes et al., 1988; 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, **(32P)**-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 H2SO4 (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, Medicin 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 Ultrasphere C-3 column (1 x 25 cm) and the indicated H2O-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 **(35S)**-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 x 107 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-wall plastic containers that were placed within unlined plastic boxes (60 x 42 x 40 cm) and kept in a growth chamber (Meraeus, Balingen, Germany) at 75% relative humidity, 25°C, 16 h (day) and at 85% 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 107 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 (107 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 cm2 of leaf area.

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