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High-level expression of a viscotoxin in *Arabidopsis thaliana* gives enhanced resistance against *Plasmodiophora brassicae*

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Abstract

Viscotoxins are a group of toxic thionins found in several mistletoe species. The constitutive CaMV-Ω promoter was used to drive the expression of the viscotoxin A3 cDNA from *Viscum album* in transgenic *Arabidopsis thaliana* C24. Lines with high viscotoxin A3 levels in all parts of the plant were selected and tested for resistance against the clubroot pathogen *Plasmodiophora brassicae*. The transgenic lines were more resistant to infection by this pathogen than the parental line.

Introduction

Several groups of basic and cysteine-rich polypeptides of plants are now being recognized as possibly involved in the defence against phytopathogens. These include thionins [for review see 8, 10, 29], lipid transfer proteins [30], and plant defensins [66]. The toxic activities of thionins have been studied for several decades. Soon after the isolation of purothionin from wheat flour [4], it was discovered that it has antimicrobial activity [64]. Such antimicrobial activity has since been demonstrated for several other thionins [11, 25, 26, 44, 65, 67], supporting the suggestion that thionins are defence proteins [25]. Further evidence for such a possible function comes from observations that the expression of several thionin genes is inducible by phytopathogenic fungi [11, 12, 20, 68, 69]. Furthermore, it has been shown that the expression of α-hordothionin in transgenic tobacco plants can lead to enhanced resistance against a phytopathogenic bacterium [16]. In similar experiments, however, Florack *et al.* [27] did not find enhanced resistance in transgenic tobacco lines expressing a synthetic α-hordothionin gene.

We have recently shown that an *Arabidopsis thaliana* thionin gene is inducible by pathogenic fungi via a signal transduction pathway different from that for PR proteins [20]. The induction of this *Thi2.1* gene by *Fusarium oxysporum* f. sp. *matthiolae* is much stronger in resistant than in susceptible ecotypes (Epple, Vignutelli, Apel and Bohlmann, manuscript submitted). Finally, overexpression of the THI2.1 thionin in the susceptible ecotype Col-2 leads to higher resistance against *F. oxysporum* f. sp. *matthiolae* [20].

Viscotoxins are a group of thionins which have been isolated and sequenced from different mistletoe species [52, 72]. Mistletoe extracts are being used in the treatment of cancer [62] and as antihypertensive. Therefore, the toxicity of viscotoxins against mammals and other biological systems has been well documented [e.g. 2, 17, 39, 50]. The toxicity of viscotoxins and other thionins has been attributed to a damaging effect on membranes [e.g. 15, 17, 39]. Like other thionins, viscotoxins are also produced as preproproteins with a molecular mass of about 15 kDa [8, 10, 29].

Four viscotoxin isoforms with six cysteine residues have been characterized from *Viscum album* leaves [46, 53, 54, 55]. Additional new viscotoxin variants were identified by a PCR-based approach [60]. The thionin domains encoded by two of these cDNAs have eight cysteine residues, in contrast to six in the classical vis-

cotoxins, and the corresponding transcripts were detected in seeds. In young mistletoe leaves, viscotoxins are produced in large amounts, but are no longer detectable in senescent leaves, indicating that the plant is reutilizing the sulfur of the viscotoxins [61]. However, a sole function as storage proteins is difficult to accept considering the large number of highly divergent viscotoxin variants. This variability is higher in the thionin domains of the viscotoxin precursors than in the acidic domain or in the signal sequence [60]. A higher mutation frequency in the thionin domain has also been found for cereal thionins and has been explained as adaptation against the constant threat by highly variable pathogens [8]. Brandenburger [13] lists only three fungal pathogens of *Viscum* species in Europe and only one fungal pathogen has been reported from the North American mistletoe, *V. articulatum* [23]. It is tempting to attribute this low number of mistletoe pathogens to a very effective defence system and to assume that thionins might play an important role in this defence.

Viscum plants, being semiparasites of trees, are difficult to grow in the laboratory and, to our knowledge, have not been transformed yet. Thus, one way to study the function of viscotoxins in defence against phytopathogens is to express viscotoxins in a heterologous system and to test the resistance of transgenic plants. As a first step, we have expressed the coding sequence of the viscotoxin A3 precursor [59] with a strong constitutive promoter in *Arabidopsis thaliana* and studied the effect of viscotoxins on the resistance against phytopathogens. It has been shown before that the expression of other putative defence proteins can lead to higher resistance against phytopathogens [1, 14, 35, 36, 41, 74]. Preliminary tests with different phytopathogenic fungi showed that the parental ecotype C24 was already resistant against *F. oxysporum* f. sp. *matthiolae* and different *Alternaria* strains. C24 was however susceptible to the root pathogen *Plasmodiophora brassicae*, a soil-borne, biotrophic pathogen with a complex life cycle. Primary infection by *P. brassicae* occurs in root hairs and leads to the development of uninucleate plasmodia. Secondary infection of cortical cells results in multinucleate plasmodia and the development of the characteristic galls [34]. Here, we report that the expression of viscotoxin A3 in *A. thaliana* gives enhanced resistance against *P. brassicae*.

Materials and methods

Cloning

The coding sequence of viscotoxin A3 was amplified by PCR from the corresponding cDNA [59] with the following primers:
vis.1: 5′-CGGAATTCCATGGAAGTTGTGAGAGGC TCC-3′
vis.2: 5′-CGCGGATCCTTAAGCATCATCAACATCT TG-3′

Primer vis.1 introduced a *Nco*I site at the start codon without altering the nucleotide sequence of the coding region, and primer vis.2 introduced a *Bam*HI site just behind the stop codon. The PCR product was digested with *Nco*I and *Bam*HI and gel-purified. The insert was cloned into the vector pSH9 [32] which was also digested with *Nco*I and *Bam*HI. The correct sequence was verified by sequencing [56]. The expression cassette was excized with *Hin*dIII and inserted in pBIN19 [5].

Growth and treatment of plants

We used the *A. thaliana* ecotype C24. For seed production plants were grown in soil in a greenhouse. For growth on MS agar [45], seeds were sterilized, sown on MS plates with vitamins (glycine 2 mg/l, nicotinic acid 0.5 mg/l, pyridoxine-HCl 0.5 mg/l, thiamine-HCl 0.1 mg/l), 1% sucrose, and 0.8% agar, stored at 4 °C for two days, and grown in a growth chamber (16 h light, 20 °C; 8 h dark, 18 °C).

Generation of transgenic Arabidopsis *lines*

The *A. tumefaciens* strain LBA4404 was transformed as described by Holsters *et al.* [31]. Integrity of the transformed plasmid was confirmed by Southern blots. Root transformation of *A. thaliana* [70] was performed as described by Huang and Ma [33]. Fifty individual primary transformants were generated. Shoots were excised and transferred to test tubes to raise seeds. Homozygous lines were generated for lines with a high expression level and further propagated to obtain enough seed material for resistance tests.

Northern blots

Plants were grown on MS agar plates. Seedlings were harvested by pouring liquid nitrogen onto the plates.

Plant material was ground in liquid nitrogen and RNA was prepared as described by Melzer *et al.* [42].

Total RNA (20 μg) was separated on denaturing 1.0% agarose gels [3]. Ethidium bromide was included to verify equal loading of RNA. After transfer to Gene Screen membranes (NEN, Beverly, MA) filters were hybridized with 10^6 cpm/ml of an oligolabelled ^{32}P-viscotoxin A3 cDNA probe [24] in HYBSOL [73].

Filters were washed for 20 min at 60 °C with 2× SSC, 0.1% SDS and then 15 min at 60 °C with 0.5× SSC, 0.1% SDS. Filters were exposed to X-Omat-AR (Kodak) films at –80 °C for one hour to six days.

Southern blots

Genomic DNA from 6-week old plants was isolated according to the method of Dellaporta *et al.* [19]. 3 μg DNA was digested with the restriction enzyme *Hin*dIII (Boehringer, Mannheim, Germany) according to the manufacturer's instructions and separated on a 0.8% agarose gel. Afterwards the DNA was transferred to Pall Biodyne A membranes (Pall, Muttenz, Switzerland) and blots were hybridized [51] with ^{32}P-labelled [24] probes. Filters were washed twice with 2× SSC, 0.1% SDS at 65 °C, once with 0.5× SSC, 0.1% SDS at 62 °C, and once with 0.1× SSC, 0.1% SDS at 62 °C, and exposed for four days. The expression cassette was detected with a viscotoxin A3 cDNA probe and copy number was analyzed with a *nptII*-specific probe.

Western blots

Plants were grown on MS agar plates as described. Plant material was ground in liquid nitrogen and 1 ml pulverized material was homogenized in 4 ml Laemmli buffer [38] and incubated at 95 °C for 10 min. Cell debris was pelleted and the supernatant was precipitated with 4 vol acetone at –20 °C for 30 min. Proteins were pelleted, dried, and dissolved in Laemmli gel loading buffer. The protein concentration was determined according to Esen [22]. Proteins (30 μg) were separated on Tricine-SDS polyacrylamide gels according to Schägger and von Jagow [57] and electroblotted onto PVDF membranes (BioRad, Glattbrugg, Switzerland). Viscotoxin A3 was detected with a polyclonal antibody raised against a viscotoxin preparation [58]. The antibody was diluted 1:2000, and the blot was developed using the Tropix Western-Light Plus chemiluminescent detection kit (Tropix, Bedford, MA).

Resistance tests

A field isolate of *Plasmodiophora brassicae* was used to infect *A. thaliana* seedlings as described by Rausch *et al.* [47] with the following modifications. *A. thaliana* plants were grown for 10 days in the greenhouse. They were then inoculated with 0.5 ml of a *P. brassicae* spore suspension in 50 mM potassium phosphate buffer pH 5.5 containing 10^7 spores/ml. After infection, plants were further cultivated in the greenhouse. Roots were harvested at 2, 3, 4, 5 and 6 weeks after inoculation. About 60 plants of each line were used per time point in one experiment. Three independent experiments was performed. For each time point the infection rate was calculated as the proportion of plants which showed macroscopically detectable hypertrophy.

Results

Generation of transgenic lines with high constitutive levels of viscotoxin A3

For the constitutive expression of viscotoxin A3 we used the expression vector pSH9 which has been shown to provide high level expression of foreign proteins in all parts of *Arabidopsis thaliana* plants [32]. The coding sequence of a viscotoxin A3 cDNA [59] was amplified with specific primers (see Materials and methods) to introduce an *Nco*I site at the start codon and a *Bam*HI site behind the stop codon without altering the encoded amino acid sequence and inserted into pSH9. The final expression cassette as shown in Figure 1 was introduced into pBIN19 [5] for root transformation [33] of *A. thaliana* C24. Kanamycin-resistant explants were regenerated and 16 transgenic lines were screened for viscotoxin expression by northern blots (Figure 2). Surprisingly, and in contrast to results obtained with the expression of crambin and barley leaf thionin DB4 (Holtorf and Bohlmann, unpublished results), the vast majority of all lines had a very uniform expression level. Only one line (No. 59) out of 16 had a very low transcript level. The reason for this is not known.

Four viscotoxin lines with a high transcript level were selected and further analyzed. Southern blots (Figure 3) revealed that all four lines had a complete expression cassette (Figure 3A) and that all of them had only a single copy of the transgene (Figure 3B). All four lines were also characterized by western blots using an antibody against viscotoxin [58]. This antibody also recognizes barley leaf thionins [59] which

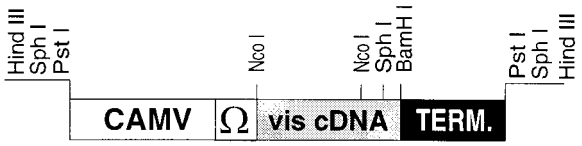

Figure 1. **Construct for the expression of viscotoxin A3.** The coding sequence for viscotoxin A3 was inserted into pSH9 carrying a CaMV promoter with Ω-element and the CaMV terminator.

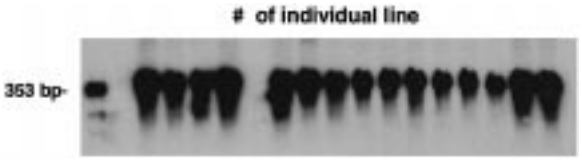

Figure 2. **Northern blot screening of the transgenic lines.** Lines depicted in bold were further screened by Southern blots and western blots. wt, wild type; vis, viscotoxin A3 cDNA insert.

were used as a control (Figure 4A). All transgenic lines, but not the wild-type C24, gave strong signals with the antibody in the region of 5 kDa, indicating that the thionin preproprotein was correctly processed to yield the mature 5 kDa viscotoxin. Two of the lines (No. 61 and No. 100) were also tested for viscotoxin expression in different parts of the plant. In both cases, roots, flowers, siliques, stems, and leaves had easily detectable viscotoxin levels (Figure 4B). Two of the viscotoxin-overexpressing lines were selected for further studies.

Resistance tests

It has been shown before that *A. thaliana* is susceptible to infection by *P. brassicae* [28, 37, 43]. Resistance tests of *A. thaliana* with *P. brassicae*, as described in Materials and methods, were performed according to a procedure established for *Brassica oleracea* [6]. The resistance of two viscotoxin-overexpressing lines after inoculation with *P. brassicae* was compared to that of the parental line C24. Plants were grown in soil in a greenhouse and infected with a spore suspension of *P. brassicae* as described in Materials and methods. For the C24 parental line susceptibility was evident from both root and hypocotyl galls (data not shown). The galls were filled with resting spores, indicating that the whole life cycle of the fungus has been completed (data not shown). Galls of the roots and the hypocotyl were also seen with the overexpressing lines, with no preference for one gall type, and the galls also contained resting spores (data not shown). However, upon closer

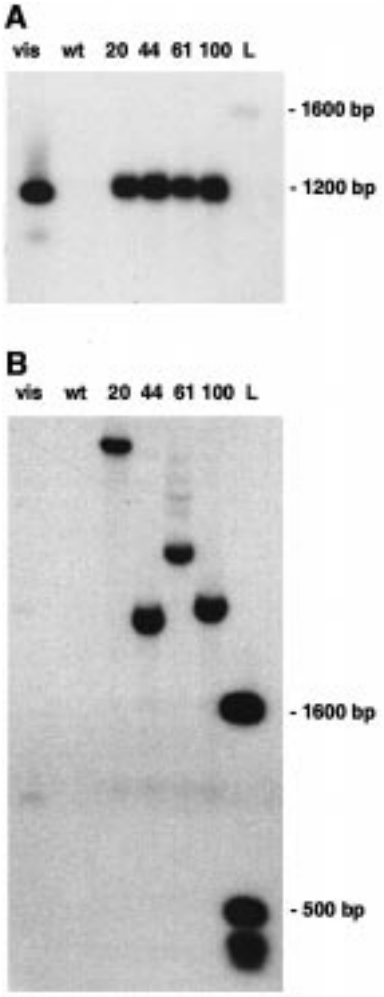

Figure 3. **Southern blot characterization of selected viscotoxin expressing lines.** Genomic DNA was digested with *Hin*dIII. A. Hybridization with a *nptII*-specific probe. B. Hybridization with a viscotoxin A3 cDNA probe. vis, viscotoxin A3 expression cassette (ca. 1200 bp); wt, C24 DNA; L, DNA marker. Numbers indicate the different transgenic lines.

inspection, it turned out that the viscotoxin lines were more resistant than the wildtype. Quantification of the infection rate in three independent experiments clearly showed that both viscotoxin-overexpressing lines were more resistant to *P. brassicae* (Figure 5). In each experiment, the infection rate at later time points was significantly lower for the viscotoxin A3-overexpressing lines as compared to the wild type and the first visible symptoms in the transgenic lines occurred later than in the wild type.

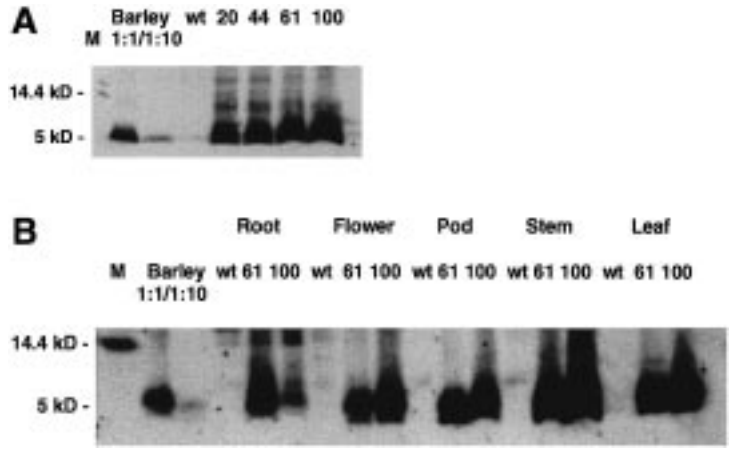

Figure 4. **Western blot analysis of selected viscotoxin-expressing lines.** A. Total protein of 4 selected viscotoxin expressing lines isolated from 4 week old plants grown on MS agar plates. B. Total protein of 2 selected lines isolated from different parts of plants grown on MS agar. M, molecular weight standards; Barley 1:1, total protein from etiolated barley seedlings; Barley 1:10, total protein from etiolated barley seedlings diluted 1:10; wt, total protein from C24 plants. Numbers indicate the different transgenic lines.

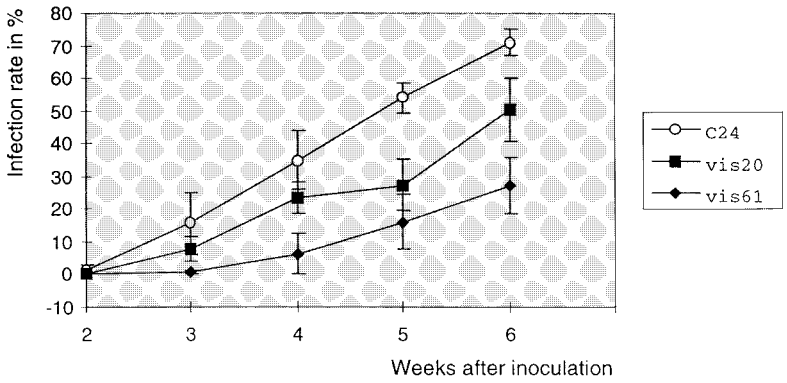

Figure 5. **Viscotoxin A3-overexpressing** *A. thaliana* **lines are more resistant against** *Plasmodiophora brassicae***.** Shown are the mean values with standard deviations of the infection rate (derived from three independent experiments) at 2 to 6 weeks after inoculation for the parental line C24 and 2 viscotoxin A3-overexpressing lines.

Discussion

We have generated transgenic *Arabidopsis thaliana* C24 lines with high levels of viscotoxin A3. The C24 ecotype was used because root explants are easily transformed [70]. Fifty lines were initially generated. Sixteen were further characterized by northern blots and four lines with the highest transcript level were selected. Southern blot analysis of the four lines indicated that all of these had single copies of the transgene integrated. This is in agreement with previous studies which have shown that single-copy lines are primarily obtained with the root transformation method used [32].

The transgenic lines contained high levels of viscotoxin and it was thus possible to detect the protein immunologically in total protein extracts. Other groups had to use enrichment procedures to detect hordothionins [16, 27] or barley leaf thionins (Mollenhauer and Apel, unpublished results) that were expressed in transgenic tobacco plants. For comparison, we included total protein extracts from etiolated barley seedlings which are known to contain very high levels of barley leaf thionins [48]. The four lines tested on western blots gave all very strong signals with an antibody directed against viscotoxin [58] that were stronger than with the barley leaf thionin control. Although the antibody does not recognize barley leaf thionins as effectively as

viscotoxins [59], we conclude from these results that all four lines have high levels of viscotoxin A3.

During the characterization of the viscotoxin-expressing lines we noted that they showed an altered phenotype when grown on MS agar under short-day conditions (Holtorf and Bohlmann, unpublished results). This is manifested as necrosis of the leaves and the apical meristem. Such phenotypes have never been observed with crambin [71] and barley leaf thionin DB4 [9] expressing lines (Holtorf and Bohlmann, unpublished results). Phytotoxicity of thionins has been described before [40, 49]. However, the transgenic lines looked quite normal when grown in soil under greenhouse conditions. Only line C24vis61 stayed small and produced only few seeds, but even this line showed a normal root growth.

The resistance of viscotoxin A3-overexpressing lines was tested against *P. brassicae*. Susceptibility of *A. thaliana* to infection by *P. brassicae* has been demonstrated before [28, 37, 43]. Susceptibility of the parental line C24 is clearly evident from hypertrophic growth of roots and hypocotyl. Furthermore, resting spores have been found in these galls, indicating a completion of the lyfe cycle. A quantification of the infection rate at different time points after infection has been done in three independent experiments with the wild type and two different viscotoxin-expressing lines. The experiments show that the transgenic lines are more resistant than the wild type. The occurrence of galls with resting spores on wild-type plants and on transgenic plants indicates that the viscotoxin might restrict the primary infection of root hairs or the first steps of secondary infections, resulting in lower infection rates. It seems that once the pathogen has established itself in the plant, viscotoxins may no longer be effective against the fungus, leading to galls of comparable size.

The mechanism by which this higher resistance against *P. brassicae* is achieved is not known yet. However, it has been shown before that thionins have *in vitro* toxicity against different phytopathogens [11, 25, 26, 44, 65, 67]. Whether the endogenous *A. thaliana* thionins play a role in the natural resistance against *P. brassicae* is not known yet. For *Hordeum vulgare* and *H. murinum* an expression of leaf thionin genes in the roots has been documented [7, 63] and Valè *et al.* [69] have recently shown that leaf thionin genes in *H. vulgare* roots are induced by infection with *Drechslera graminea*. Resistance in *Brassica napus* and *B. campestris* is mainly monogenic but multigenic in *B. oleracea* [18]. Recently, a resistance gene against *P. brassicae* has been identified in *A. thaliana* [28]. The use of such specific resistance genes to control clubroot disease in cruciferous crops is, however, restricted by the occurence of many different pathotypes. The variability of the pathogen will easily overcome a defence that depends on a single resistance gene. Other approaches are therefore needed which could effectively control clubroot disease. Our results indicate that this could be achieved by the expression of heterologous defence proteins such as thionins.

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