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Sporamin-mediated resistance to beet cyst nematodes (*Heterodera schachtii* Schm.) is dependent on trypsin inhibitory activity in sugar beet (*Beta vulgaris* L.) hairy roots

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Abstract

Sporamin, a sweet potato tuberous storage protein, is a Kunitz-type trypsin inhibitor. Its capability of conferring insect-resistance on transgenic tobacco and cauliflower has been confirmed. To test its potential as an anti-feedant for the beet cyst nematode (*Heterodera schachtii* Schm.), the sporamin gene *SpTI-1* was introduced into sugar beet (*Beta vulgaris* L.) by *Agrobacterium rhizogenes*-mediated transformation. Twelve different hairy root clones expressing sporamin were selected for studying nematode development. Of these, 8 hairy root clones were found to show significant efficiency in inhibiting the growth and development of the female nematodes whereas 4 root clones did not show any inhibitory effects even though the *SpTI-1* gene was regularly expressed in all of the tested hairy roots as revealed by northern and western analyses. Inhibition of nematode development correlated with trypsin inhibitor activity but not with the amount of sporamin expressed in hairy roots. These data demonstrate that the trypsin inhibitor activity is the critical factor for inhibiting growth and development of cyst nematodes in sugar beet hairy roots expressing the sporamin gene. Hence, the sweet potato sporamin can be used as a new and effective anti-feedant for controlling cyst nematodes offering an alternative strategy for establishing nematode resistance in crops.

Introduction

Plant parasitic nematodes are serious pests in many crops worldwide. Total annual economic losses caused by plant-parasitic nematodes are estimated to be in the range of USD 77 billion (Sasser *et al.*, 1987). Economically most relevant are sedentary endoparasites of the genera *Heterodera* and *Globodera* (cyst nematodes), and of the genus *Meloidogyne* (root-knot nematodes). They represent the most advanced level of root parasitism as they induce and maintain specific nurse cell structures such as giant cells and syncytia as a continuous source of food for development and reproduction (Jung and Wyss 1999). The use of nematode resistant cultivars is an important alternative to fight plant parasitic nematodes in the field in relation to chemical control which relies on highly toxic substances.

In the past four years, three nematode resistance genes have been cloned from sugar beet, tomato and potato (Cai *et al.*, 1997; Milligan *et al.*, 1998; van der Vossen *et al.*, 2000). Those genes have been successfully used for breeding of resistant varieties by conventional means. However, resistances which rely on gene-for-gene relationships can easily be overcome by virulent pathotypes. Therefore, a 'technical' resistance relying on anti-feeding strategies is an interesting alternative.

Two principal strategies are under discussion. One is aiming at the breakdown of the specific feeding structures by the introduction of genes encoding phytotoxic compounds that disrupt and disintegrate the

feeding cells (Goddijn *et al.*, 1993). This can only be successful if promoters are available which are exclusively active in feeding cells (Gurr *et al.*, 1991; Atkinson and Hepher, 1996). The limited availability of non-leaky promoters is still the main obstacle to this strategy.

Alternatively, genes are expressed in plants whose products are non-phytotoxic but either toxic to the nematode or reducing the digestibility of the nutrients taken up from the feeding cell, such as plant proteinase inhibitors (PIs). PIs are an important element of natural plant defence strategies (Ryan, 1990). Until now, numerous PIs have been identified which target all four classes of plant proteinases, cysteine, serine-metallo and aspartyl (Richardson, 1991). Those inhibitory proteins have been shown to reduce the capacity of certain insects to use dietary protein resulting in growth retardation and reduced fecundity (Hilder *et al.*, 1987; Ryan, 1990; Abhay *et al.*, 1999). Thus far, many reports have demonstrated that PIs expressed in plants reduce the rate of insect development, for example, cowpea trypsin inhibitor (CpTI) on lepidopteran insect *Heliothis virens* in tobacco plants (Hilder *et al.*, 1987), potato inhibitor II (PI-II) on *Spodoptera exigua* in tobacco (Johnson *et al.*, 1989), and sweet potato trypsin inhibitor (SpTI) on *Spodoptera litura* in tobacco and *Brassica* spp. (Yeh *et al.*, 1997b; Ding *et al.*, 1998).

Both serine and cysteine proteinases are present in plant parasitic nematodes (Koritsas and Atkinson, 1994; Lilley *et al.*, 1997). Their activities have been detected in the nematode intestine where they are involved in digestion of dietary proteins (Lilley *et al.*, 1996). In particular, PIs are of interest as anti-feedants against nematodes because of their low molecular weight. Molecular weight is an important factor because nematodes take up their nutrients via the feeding tube. In the case of the beet cyst nematode (*Heterodera schachtii*), Böckenhoff and Grundler (1994) determined the exclusion size for taking up molecules via the feeding tube to be in the range of 40 kDa. Inhibitory effects of PIs on nematodes have been reported. Early growth and sexual fate of the potato cyst nematode *Globodera pallida* were negatively affected after feeding from transgenic potato plants expressing *CpTI* (Atkinson and Hepher 1996). The rice oryzacystatin-1 (*Oc-1*) expressed in transgenic tomato hairy roots showed detrimental effects on growth and development of *G. pallida* (Urwin *et al.*, 1995). A modified rice cystatin, Oc-1ΔD86, produced in transgenic *Arabidopsis thaliana* had a profound effect on the size and fecundity of females from *H. schachtii* and *Meloidogyne incognita* (Urwin *et al.*, 1997). Furthermore, an enhanced resistance to those nematodes could be achieved by co-expressing (pyramiding) two distinct TIs, CpTI and Oc-1ΔD86 in *Arabidopsis* plants (Urwin *et al.*, 1998) broadening the potential of PIs for nematode control. Recently, a cysteine proteinase inhibitor expressed in potato plants provided the first demonstration that transgenic resistance to nematodes such as the potato cyst nematode *Globodera pallida* can be effective under field conditions (Urwin *et al.*, 2001).

Sporamins are tuberous storage proteins and account to 80% of soluble protein in sweet potato tubers (Maeshima *et al.*, 1985). Sporamin genes belong to a gene family with more than 10 members. Based on nucleotide homology they can be grouped into two gene subfamilies, sporamin A and B (Hattori *et al.*, 1989). A full-length sporamin cDNA, referred to as *SpTI-1*, was isolated from a sweet potato tuberous cDNA-library. It encodes a proprecursor with an N-terminal extension composed of a 21 amino acid signal peptide, a 16 amino acid propeptide and a 23 kDa mature protein. *SpTI-1* belongs to the sporamin A subfamily. In potato, under non-stress conditions, *SpTI-1* is transcribed only in tuber cells. However, transcription can be activated in leaves by wounding stress (Wang *et al.*, 2002). In addition, the SpTI-1 product functions as a serine-protease-inhibitor (Kunitz-type) with trypsin-inhibitory activity (Yeh *et al.*, 1997a; Yao *et al.*, 2001). Its defensive role in protecting plants from herbivorous damage was confirmed in transgenic tobacco which after transformation with the *SpTI-1* gene displayed resistance against tobacco cutworm (*Spodoptera litura*; Yeh *et al.*, 1997b). In another study, the *SpTI-1* gene was expressed in Taiwan cauliflower (*Brassica oleracea* var. *botrytis* L.) cultivars, which resulted in transgenic plants showing resistance to *Spodoptera spp.* (Ding *et al.*, 1998). Until now, there is no report about any inhibitory effects to plant parasitic nematodes.

In this study, the sporamin gene *SpTI-1* was expressed in sugar beet hairy roots. After inoculation with the beet cyst nematode the efficiency of sporamins in inhibiting the development of cyst nematodes in sugar beet hairy roots was demonstrated.

Materials and methods

Plant materials, hairy-root culture and GUS-reporter gene assay

The susceptible sugar beet line 93161p, kindly provided by the breeding company A. Dieckmann-Heimburg (Nienstädt, Germany) was used for the generation of hairy roots. Leaf stalks were sterilized by submergence in calcium hypochloride (5%) for 10 min, followed by treatment with 70% alcohol for 5 min, and repeated washing in sterile double-distilled H_2O. The sterilized leaf stalks were cut into pieces of 2 cm length and incubated with *A. rhizogenes* as described below for 10 min. The infected explants were soaked on sterilized filter paper before cultivation on solid half-strength ($^1/_2$) B5 medium (Gamborg *et al.*, 1968). After 2 days of co-cultivation in the dark, explants were transferred to $^1/_2$ B5 medium containing 400 μg/ml cefotaxime to eliminate *A. rhizogenes*, and incubated at 26 °C under a 16 h light/8 h dark cycle. Single hairy roots 1 cm long were excised and subcultured on $^1/_2$ B5 medium containing 150 mg/l cefotaxime under the same conditions. Prior to nematode infection, histochemical GUS tests were performed. Pieces of hairy roots 1 cm in size were incubated in 50 mM sodium phosphate pH 7.0, 2 mM X-Gluc for 16 h at 37 °C according to Jefferson (1987).

Plasmid constructs and Agrobacterium *culture*

The full-length sporamin cDNA *SpTI-1* (0.9 kb) including two N-terminal signal sequences was cloned as an *Eco*RI fragment into the *Eco*RI site of the binary vector pAM194 under the control of the CaMV35S promoter. The binary vector pAM194 kindly provided by Planta (Einbeck, Germany) was constructed by introducing a GUS intron gene into the *Hin*dIII site of the pRT104 vector (Töpfer *et al.*, 1987) under the control of the 35S promoter. The resulting plasmid pAM/SpTI-1 was confirmed by sequencing (Li-Cor sequencer, MWG, Ebersberg, Germany), and electroporated (Gene pulser II, BioRad, Hercules, USA) into cells of *A. rhizogenes* strain AR15834 which harbours a wild-type plasmid pRi15834 as described by Kifle *et al.* (1999). Transformed *Agrobacterium* cells were grown on 2YT medium containing 50 mg/l kanamycin and 100 mg/l rifampycin overnight. The overnight cultures were used to inoculate 150 ml 2YT medium without antibiotics and grown to an OD600 of ca. 0.4–0.6. The bacterial cultures were centrifuged at 4000 × *g*, 4 °C for 10 min and bacteria were re-suspended in 15 ml 2YT medium ready for transformation of sugar beet leaf stalks.

Nematode resistance tests

Inoculation of hairy roots with nematodes was performed according to the protocol described by Sijmons *et al.* (1991). A total of 300 sterile infective juveniles of the beet cyst nematode (*H. schachtii* Schm.) were added to each culture plate. At 2–3 weeks after infection, developing females were observed under a stereomicroscope. The number of developed females was determined 4 weeks after infection. The penetration rate of nematodes into the hairy roots was estimated 1 week after inoculation according to Grundler *et al.* (1991).

Southern analysis, northern analysis and RT-PCR

Hairy roots were harvested two weeks after subcultivation for isolation of genomic DNA, total RNA and total protein. The same sample was used for resistance tests. Genomic DNA was extracted from hairy roots as described by Rogers and Bendich (1985). After digestion with *Xho*I or *Eco*RI, 5 μg DNA samples were separated on 1.0% agarose gels and transferred onto Hybond-N+ membrane (Amersham Pharmacia Biotech, Freiburg, Germany) by capillary diffusion blotting overnight, with 0.25 M NaOH/1.5 M NaCl as blotting solution. Total RNA was extracted from hairy roots with the Trizol method (Gibco-BRL, Life Technologies, Karlsruhe, Germany). A 20 μg aliquot of total RNA was electrophoresed on 1.3% agarose-formaldehyde gels and transferred onto Hybond-N+ membrane with 20× SSC. The α-^{32}P-labelled DNA probe for *SpTI-1* was generated according to Feinberg and Vogelstein (1983). Hybridization of the membranes, washing steps and signal detection were performed as described by Pillen *et al.* (1992). The 18S rRNA probe was hybridized as a control for RNA loading. Messenger RNA was purified from 1 μg total RNA by using the Oligotex mRNA mini kit (Quiagen, Hilden, Germany). First-strand cDNA was synthesized in a 20 μl reaction volume with the SuperScript First-Strand Synthesis System (Gibco-BRL, Life Technologies, Karlsruhe, Germany). RT-PCR was performed with 2 μl of reverse transcription products as template and *SpTI-1*-specific primers (Yeh *et al.*, 1997a) for 35 cycles (94 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s). The non-transcribed mRNA served as a control to confirm absence of genomic DNA.

Western blot analysis

A 10 mg portion of hairy roots were ground in a mortar with 600 μl extraction buffer (10 mM Tris-HCl pH 7.5, 1mM EDTA, 1mM PMSF, 1% DTT), then centrifuged at 10 000 $\times$ *g* for 10 min at 4 °C. The crude extract of total soluble protein was obtained from the supernatant. Protein samples were quantified (Brandford, 1976) and 10 μg total protein were separated by 12.5% SDS-PAGE and blotted onto PVDF membranes (Immobilon, Millipore, Eschborn, Germany). Immunological reactions were performed with anti-sporamin antiserum (Yeh *et al.*, 1997a) and AP-conjugated secondary antibody following the supplier's protocol (BM Chromogenic Western Blotting Kit, Roche Diagnostics, Mannheim, Germany).

Assay for trypsin inhibitory activity of sporamin

The quantitative assay of trypsin inhibitory activity of hairy roots was performed mainly according to the protocol described by Ding *et al.* (1998). Trypsin (1 μg) and total protein of hairy roots (200 μg) were mixed together in a 800 μl buffer (0.1 M Tris-HCl pH 8, 0.01 M $CaCl_2$) and to react for 5 min at 37 °C. Then 5 μl of DL-BAPA (500 mg/ml DMSO) was added and incubated for further 20 min at 37 °C. The reaction was stopped with 250 μl of 10% acetic acid. Remaining trypsin activity was measured with OD410. Trypsin inhibitory activity (%) was calculated with the following equation: trypsin inhibitory activity (%) $= [(OD_{standard} - OD_{sample})/OD_{standard}] \times 100$, where $OD_{standard}$ is the reaction of trypsin and DL-BAPA without total protein of hairy roots, and OD_{sample} is the reaction of trypsin and total protein of hairy roots and then DL-BAPA.

The assay for trypsin inhibitory activity on the SDS-polyacrylamide gel was performed with a modified method described by Chan and Delumex (1982). The crude extracts of protein samples from hairy roots were dissolved in the sample buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% sucrose, 10 mM ascorbic acid, 1 mM PMSF, 2 mM DTT but without addition of 2-mercaptoethanol and without boiling. The protein concentration was determined as described above. 200 μg total protein was electrophoresed on 10% native SDS-PAGE gel. After electrophoresis, the gel was shaken gently in a 25% v/v 2-propanol/10 mM Tris-HCl pH 7.4 solution for 30 min to remove SDS, and then in 10 mM Tris-HCl pH 8.0 for another 30 min to renature the proteins. The gel was incubated in a trypsin solution containing 40 μg/ml bovine trypsin (Sigma, Munich, Germany) in 50 mM Tris-HCl pH 8.0, 50 mM $CaCl_2$ for 40 min before being transferred to a freshly prepared substrate-dye solution, which consisted of 2.5 mg/ml N-acetyl-DL-phenylalanin β-naphthyl ester (APNE, Sigma, Munich, Germany) with dimethylformamide (DMF) and tetrazotized *O*-dianisidine dye solution (0.5 mg/ml in 50 mM Tris-HCl pH 8.0, 50 mM $CaCl_2$). The gel was incubated for 25 min at room temperature for colour development. Acetic acid (10% v/v) was added to stop the reaction. Clear zones on the gel were indicative for proteins with trypsin inhibitory activity. The area of the zone, correlating with the trypsin inhibitory activity level, was measured with the software Quantity One (BioRad, Munich, Germany).

Results

Generation of SpTI-*transgenic hairy roots from sugar beet*

Hairy roots were produced after inoculation of leaf stalks with *A. rhizogenes* harbouring the pAM/SpTI-1 plasmid. As a control, hairy roots were produced with *A. rhizogenes* containing the empty pAM194 vector only. Hairy roots emerged from explants 5–7 days after inoculation. Root tips were then separated and subcultured in the dark at 24 °C for further growth giving rise to clonal root cultures. Altogether, 40 independent hairy root clones were obtained after transformation with the pAM/SpTI vector construct and 32 root clones with the pAM194 control. To identify those roots that were co-transformed with both Ri plasmid and pAM194 vector, all hairy roots were subjected to GUS tests. As a result, 24 out of 40 pAM/SpTI root clones were found to be GUS-positive, which suggests that they carried the whole insert including the sporamin gene. To confirm that the *SpTI-1*-gene is expressed in pAM/SpTI-transgenic roots, RT-PCR with *SpTI-1* gene-specific primers was carried out. Of the 24 GUS-positive root cultures, 12 gave a clear PCR product with 650 bp in size which is in accordance with the expected fragment size (data not shown). In addition, root cultures were also inspected under the microscope and there were no pronounced differences in growth rates and morphology of hairy roots expressing the sporamin compared to roots transformed with the empty pAM194 vector. Therefore, the 12 RT-PCR-positive hairy roots were employed for further nematode inoculation experiments together with

6 randomly selected pAM194 roots which served as a control.

Nematode inoculation experiments

From each of the 12 pAM/SpTI hairy roots, 8 subclones were produced. Each clone was inoculated with about 300 J2 juveniles and incubated in the dark at 22 °C. Hence, the inoculation experiments were repeated 8-fold. In the same manner, the six pAM194 roots were inoculated. The average penetration rates varied between 20% and 23% of all inoculated J2 juveniles, but there was no obvious difference in penetration rate between pAM/SpTI roots and control roots (Figure 1). Four weeks after inoculation, the developing females were counted under the microscope. On the control roots, nematodes were able to induce the formation of syncytia and develop regularly into males and females. On average, 23 adult females per plate were counted 4 weeks after inoculation (Figures 1 and 2D), which is in accordance with the result reported by Kifle *et al.* (1999). However, a significant reduction of developed females was observed for sporamin expressing root clones 32, 31 and 17 as compared to control roots (Figure 1), which are henceforth referred to as infection response group 1. Although a few juveniles were able to further develop into adult females those were much smaller in size than females on control roots and they did not contain eggs (Figure 2B). A similar effect was observed on root clones 49, 55, 35, 20 and 61, on which a small proportion of juveniles developed into normal females but with a strong reduction of female number relative to control roots (Figure 1, group 2). In contrast, no inhibitory effect could be observed on root clones 14, 52, 69 and 63 (Figure 1, group 3), where most of the nematode juveniles developed regularly into females. Microscopic observation on the hairy roots of the group 1 showing nematode inhibition (clones 32, 31 and 17) revealed that the J2 juveniles regularly proceeded into the vascular cylinder of the root to initiate formation of syncytia, but after that further development stagnated at the early J2/J3 stage. Also, disruption of the feeding sites and strong necrosis around the feeding sites were observed (Figure 2A), probably due to the disability of the nematode to maintain the function of the feeding cells.

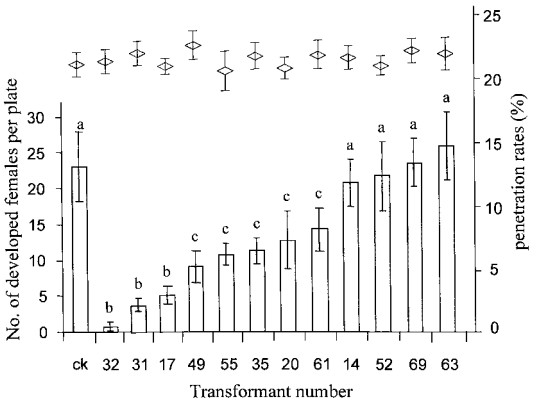

Figure 1. Results from nematode resistance tests with hairy roots transformed with the *SpTI-1* gene and inoculated with *H. schachtii* juveniles. ck, hairy roots transformed with the pAM194 vector as a control. The numbers of developed females are given as mean values (column) of 8 independent tests. Error bars equal one standard deviation. Significantly different means are indicated by different letters (a, b, c). The Tukey test was performed at a probability level of <0.01. For each clone, the average penetration rate of the inoculated J2 is shown as mean value (rhombs) of 4 independent tests with the standard deviation.

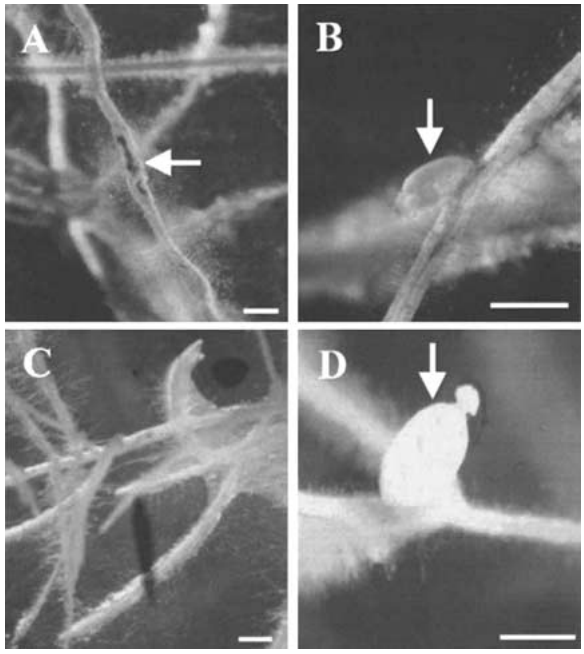

Figure 2. Microphotographs of roots expressing the sporamin *SpTI-1* gene after nematode infection. Hairy roots transformed with the *SpTI-1* gene 5 days (A) and 4 weeks (B) after nematode inoculation when nematodes could be observed only in rare cases. The picture shows a nematode with staggered growth and without eggs. Hairy roots transformed with the empty pAM194 served as controls 5 days (C) and 4 weeks (D) after nematode inoculation. Necrosis at the point of nematode infection (2A) and developing nematodes (2B, 2D) are marked by an arrow. No necrosis was obvious in the control roots (2C) where females containing eggs could develop regularly. The bar represents 400 μm.

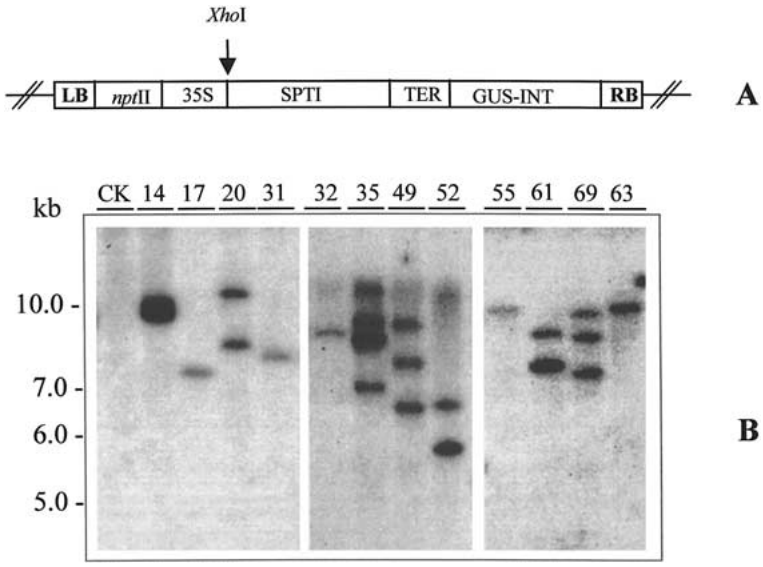

Figure 3. Genomic DNA blot analysis of *SpTI-1*-transgenic hairy root transformants. A. The *Xho*I recognition site within the pAM/SpTI vector. B. Determination of copy numbers of the integrated *SpTI-1*-gene. Genomic DNA (10 μg) was digested with *Xho*I, separated in 0.75% agarose gels and probed with the *SpTI-1*-gene after Southern transfer. CK, hairy root cultures transformed with the pAM194 as a control; 14–69, *SpTI-1* transgenic hairy roots that showed varying nematode inhibiting efficiency. Molecular size markers are indicated at the left in kb. Exposure time: 48 h.

Copy number, transcription and translation of the SpTI-1 *gene in transgenic root cultures*

The nature of various efficiencies of hairy roots expressing the *SpTI-1* gene in inhibiting the growth and development of beet cyst nematodes was analysed with respect to their level of *SpTI-1* gene expression and their anti-trypsin activity. The copy number of the *SpTI-1* gene integrated into the sugar beet genome was determined by Southern analysis. The genomic DNA of the 12 selected pAM/SpTI-root clones and one control root was digested with the restriction enzyme *Xho*I, which cuts the upstream region of the gene *SpTI-1* within the construct pAM/SpTI once (Figure 3A). The *SpTI-1* probe could hybridize only with one restriction fragment covering the right border site. As a result, the hairy root clones 17, 31, 32, 55 and 63 yielded one band, whereas the hairy root clones 14, 20, 52 and 61 showed two bands, clones 49 and 69 gave three bands, and clone 35 revealed four bands representing one, two, three and four insertions of the *SpTI-1* gene in the sugar beet genome, respectively (Figure 3B). No hybridization signal was visible with genomic sugar beet DNA (Figure 3B).

The transcriptional activity of the *SpTI-1* gene in hairy roots was determined by northern blot hybridization. Total RNA, isolated from the selected 12 hairy roots and one randomly selected control root, was hybridized with the *SpTI-1*-probe. As shown in Figure 4, a transcript of about 1.1 kb was present in all 12 root cultures but not in the control root (Figure 4A). The transcription levels varied slightly among the root cultures with no obvious correlation with copy numbers of the *SpTI-1* gene.

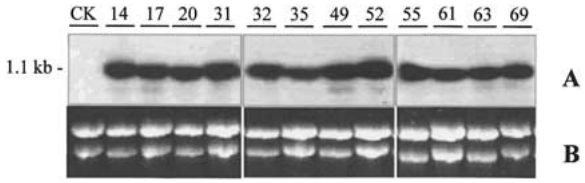

Figure 4. Determination of *SpTI-1* gene expression by northern analysis in transgenic hairy roots. A. A 20 μg portion of total RNA was loaded in each lane and probed with the *SpTI-1* gene. B. The ethidium bromide-stained rRNA served as a loading control. ck, hairy root cultures transformed with the empty pAM194 vector; 14–69, *SpTI-1*-transgenic hairy root clones. Exposure time: 48 h.

Total soluble protein was extracted from the hairy root cultures and western blots were probed with anti-SpTI-1 antiserum. A 23 kDa band, corresponding to the SpTI-1 mature protein, was detectable in all hairy roots carrying the *SpTI-1* gene but not in the control roots (Figure 5A). The accumulation of SpTI-1 protein varied among the root cultures (Figure 5A) without a correlation with transcriptional activity of the *SpTI-1* genes. These observations demonstrated that the

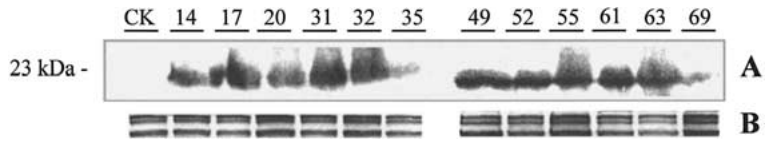

Figure 5. Western analysis of *SpTI-1*-transgenic hairy roots. A. A 10 μg portion of total protein was separated by SDS-PAGE and blotted onto a PVDF membrane. An immune reaction was elicited with anti-sporamin antiserum. ck, hairy root cultures transformed with the empty pAM194 vector; 14–69, transgenic hairy root clones. B. Part of the Coomassie blue-stained SDS-polyacrylamide showing that equal amounts of protein had been loaded on the gel.

SpTI-1 protein was produced in root cultures belonging to different infection response groups. Moreover, both transcriptional and translational activity of the sporamin gene did not correlate with their nematode inhibition efficiency.

Trypsin inhibitory activity assays

It is well known that the extent of trypsin inhibitory activity is directly related to anti-insect capability and the activity level is a critical factor for insect resistance efficiency as well (Yeh *et al.*, 1997b). Therefore, an anti-trypsin activity assay was performed on the selected pAM194/SpTI transgenic roots and the control roots. The relative trypsin inhibitory activity of hairy roots was determined quantitatively according to the protocol described by Ding *et al.* (1998). Trypsin inhibitory activity was measured in all pAM/SpTI transgenic roots but not in control roots. A strong variation of the activity levels was observed among the pAm194/SpTI roots, which correlated with nematode-inhibitory efficiency (Figure 6A) but not with sporamin level. The regression line in Figure 6 demonstrates the relationship between nematode-inhibitory effect and trypsin-inhibitory activity of the hairy roots expressing the sporamin gene.

In addition, trypsin-inhibitory activity of 4 selected hairy root clones (Nos. 17, 32, 61 and 63) was demonstrated after separating aliquots of total protein samples (200 μg) in 12% SDS-polyacrylamide gels. Gels were stained according to the method described by Chan and Delumex (1982). The clear zone on the gel represents the proteins with trypsin-inhibitory activity (Figure 7A). Trypsin-inhibitory activity was clearly detectable in hairy root clones belonging to infection response groups 1 and 2 (Nos. 17, 32 and 61), but it was absent from control roots and from hairy roots belonging to infection response group 3 (e.g. No. 63). Furthermore, the activity level of the hairy root clone 32 was about 3-fold higher than that of the hairy root clone 17, and about 5-fold higher than that of the hairy root clone 61 as measured with the software Quantity One (BioRad), which is also in correspondence with the nematode-inhibiting efficiency of those root clones.

Discussion

After invading the vascular cylinder of the host root, cyst nematodes trigger the formation of specific feeding cells. Proper development of those cells is crucial for regular development of larvae into adults. The targeted deletion of those cells offers the possibility for fighting the parasite without side effects upon plant development. The effective use of anti-nematode genes which cause disruption of feeding cells to generate artificial resistance relies on their targeted expression or non-phytotoxicity to the host. Thus, compounds produced by the plant itself will be the most promising ones. In this study, we expressed the sweet potato sporamin, a trypsin inhibitor, in sugar beet hairy roots to test its efficiency for controlling nematode infection.

Twelve *SpTI-1*-transgenic sugar beet hairy root clones showed variation in their response to nematode infection. Of these, three displayed high, three medium and another four no inhibitory effects, although the *SpTI-1* gene was regularly transcribed and translated in all 12 hairy roots. We did not find any indication for down-regulation of the CaMV 35S promoter in nematode feeding sites as has been reported previously (Goddijin *et al.*, 1993). Although feeding-site-specific expression has not been determined here, the nematode inhibitory effects suggest strong activity of the sporamin gene even within the feeding cells. Several lines of evidence demonstrate that the inhibition of nematode development is due to the activity of the sporamin gene. Enzyme activity experiments demonstrated that the trypsin inhibitory activity was a critical factor for nematode growth inhibition. A negative correlation was found between trypsin inhibitory activity and nematode development as determined by the number of females. It is interesting to note that the amount of the SpTI-protein as judged from western

analysis varied upon hairy roots, but was not consistent with the level of anti-trypsin or anti-nematode activity. As for root clone 63, the SpTI-1-protein was present but almost no trypsin inhibitory activity could be detected which explains the lack of resistance to nematode infection. The reason for this is yet unknown, but post-translational modifications observed in transgenic plants (Jentoft *et al.* 1990), such as glycosylation of proteins, are most likely to affect the activity of the sporamin. Also, folding of proteins to correct secondary structure may play an important role for inhibitory activity (Song *et al.*, 1999). Site-directed mutagenesis gave evidence that a negatively charged trypsin inhibitory loop in sporamin is a critical structure, which comprises the active site interacting with trypsin and blocking its enzymatic function (Yao *et al.*, 2001). Our observations are in accordance with the report by Yeh *et al.* (1997b) who described that insect resistance of transgenic tobacco plants expressing sporamin correlated with the anti-trypsin activity of sporamin, but an unknown post-translational modification caused the complete loss of the trypsin-inhibitory effect in transgenic tobacco. Therefore, *Spodoptera litura* was able to feed on those transgenic tobacco plants as on untransformed controls (Yeh, unpublished results). For some reasons, it will be important to understand these modification processes for improving and optimizing inhibitory effects of sporamins on nematode development. In fact, Urwin *et al.* (1997) have successfully demonstrated that transformation of *Arabidopsis* with an engineered rice cystatin, Oc-1ΔD86, resulted in proteins with much higher proteinase inhibitory activity leading also to a profound inhibitory effect on growth and development of *H. schachtii* and *M. incognita*, and enhanced resistance to the nematodes could be achieved by co-expressing (pyramiding) two distinct TIs (Urwin *et al.*, 1998).

The number of adult females proved to be a reliable index of resistance to the beet cyst nematode (Cai *et al.*, 1997; Kifle *et al.*, 1999). Also, inhibitory effects of PIs on nematode growth and development of females have been well documented before. Transgenic potato plants expressing CpTI retarded early growth and sexual fate of the potato cyst nematode *G. pallida* (Atkinson and Hepher, 1996). The rice oryzacystatin-1 as well as a modified rice cystatin, Oc-1ΔD86, expressed under the control of the CaMV 35S promoter in tomato hairy roots and *Arabidopsis* plants showed marked inhibitory effects on the size and also fecundity of females of *G. pallida, H. schachtii* and

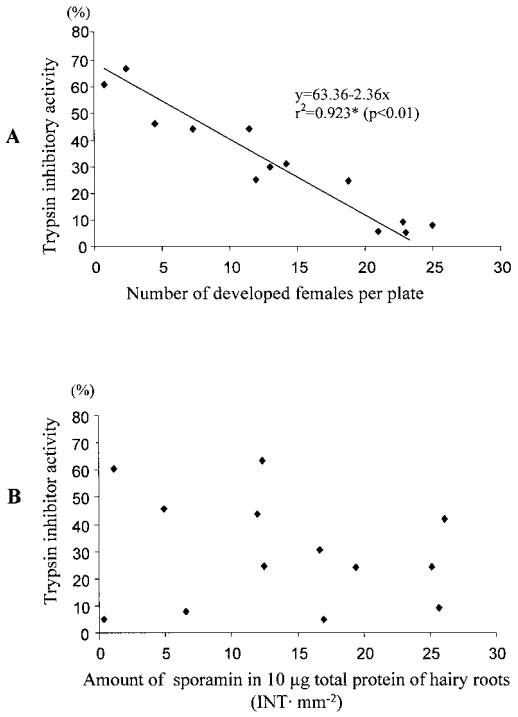

Figure 6. Relationship between trypsin inhibitor activity and (A) nematode development and (B) sporamin concentration. Regression curve between sporamin activity and nematode growth retardation determined on 12 hairy root clones expressing the *SpTI-1* gene ($r^2 = 0.923$). Significance was determined by the F-test. Values are means of 8 repeats. Sporamin levels were determined by densitometric measurement of western blots.

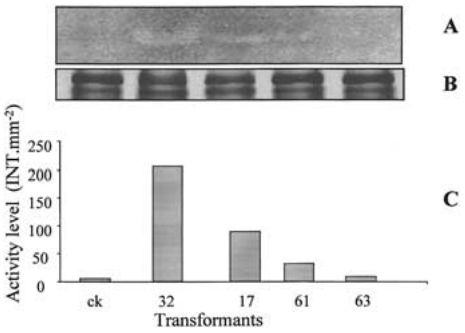

Figure 7. Trypsin inhibitory activity assay of *SpTI*-1-transgenic hairy roots. A 200 μg portion of total protein was loaded in each line. A. Trypsin inhibitory activity assay in a native SDS-polyacrylamide gel. Clear zones within the gel are indicative of sporamin with trypsin inhibitory activity. B. Part of the Coomassie blue-stained protein gel as loading control. C. Quantification of trypsin inhibitory activity of sporamin expressed in sugar beet hairy roots as determined with the software Quantity One (BioRad). ck, hairy root cultures transformed with the empty pAM194 as control; 32, 17, 61 and 63, *SpTI-1*-transgenic hairy roots.

M. incognita (Urwin *et al.*, 1995, 1997), respectively. Therefore, it was reasonable to focus in the present study on the development of the female nematodes on hairy roots. Microscopic observations demonstrated that the early development of nematodes on sugar beet hairy roots with the functional SpTI-1 protein differed from those on the control roots. In case of root clone 32, J2 juveniles were able to proceed to the vascular cylinder and initiate formation of feeding sites, but they were not able to develop regularly into females. They stagnated at the stage of J2/J3 which led to disruption of initially established feeding structures causing strong necroses around the nematode feeding sites. Obviously, sporamin is becoming effective at early stages of nematode development which is in accordance with observations on roots transformed with other PIs described above (Urwin *et al.*, 1997). A few nematode juveniles could escape from the inhibitory effect of the PI to develop into females, but in most cases they were not able to reach the size and shape required for egg production. We have observed a similar resistance response in sugar beet roots expressing the $Hs1^{pro-1}$-gene for nematode resistance which encodes a protein that is anchored in the plant cell membrane suggesting that it is not ingested by the nematode (unpublished results). Roots exhibited strong necrosis after infection with the beet cyst nematode and the nematode development was significantly inhibited resulting in a complete absence of fully developed females (Cai *et al.*, 1997). Disruption of the feeding cell structure yielded in the formation of specific membrane aggregations that condensed to electron-dense bodies filling large parts of the syncytium (Holtmann *et al.*, 2000).

Both serine proteinases, the target of *SpTI-1*, and cysteine proteinase have been found in plant parasitic nematodes such as *G. pallida* and the cyst nematode *H. glycines* (Koritsas and Atkinson, 1994; Lilley *et al.*, 1997). Their activities have been localized to the intestine of the soybean cyst nematode *H. glycines* where they are involved in digestion of dietary proteins (Lilley *et al.*, 1996). It can be expected that such proteinases are also present in *H. schachtii*. The size of the effector molecule is an important consideration in an anti-feedant defence strategy. It has been demonstrated that the loss of proteolytic activity in *H. schachtii* taken from Oc-1ΔD86-expressing *Arabidopsis* correlated with uptake of Oc-1ΔD86, a globular protein of ca. 11.2 kDa by this nematode (Urwin *et al.*, 1997, 1998). Microinjection of fluorescence-labelled dextrans of different molecular weights into the syncytia showed that the exclusion size for proteins taken up via the feeding tube is <40 kDa because only dextrans of molecular masses between 3 and 40 kDa could be ingested by the nematodes (Böckenhoff and Grundler, 1994). Therefore, it can be assumed that the *SpTI-1* gene which encodes a 23 kDa soluble protein can be taken up through the feeding tube and the stylet and delivered to within the nematode where it can exhibit effective inhibition. Alternatively, the sporamin could inhibit essential trypsin activity within the feeding cell. In this case, a serine proteinase secreted by the nematode is the potential target for sporamin.

Hairy roots have been well established as a rapid test system for studying foreign gene expression in the case of host-parasite interaction between the beet cyst nematode and sugar beet. The host-parasite interactions which had been described for whole plants carrying the $Hs1^{pro-1}$-gene were well maintained in sugar beet hairy roots (Cai *et al.*, 1997; Kifle *et al.*, 1999). The results of the present study clearly support the hypothesis that sporamin genes can be used to breed for nematode resistance in sugar beet or other crop plants although it can be expected from our results that a certain proportion of transformants expressing the gene will not show resistance. However, this does not cause major problems since during breeding strong selection among primary transformants is usually applied. The resistance of whole plants expressing the sporamin gene could not be investigated so far, because sugar beet is recalcitrant to any kind of transformation. A protocol based on transformation of guard cells has been presented (Hall *et al.*, 1996), but routine application of this technique is still a problem. With regard to risk assessment of transgenic plants, sporamin seems to be favourable. As a vacuolar storage protein (Yeh *et al.*, 1997a) it accounts for about 60–80% of total soluble protein in sweet potato. Sporamin has been demonstrated to be harmless to human beings and animals after cooking the potatoes (Yeh *et al.*, 1997b). This will enhance the potential for introducing this gene into crop plants even for human consumption. Experiments are on the way to produce transgenic beets expressing the *SpTI* gene either under the control of a constitutive promoter or under the control of a nematode-responsive promoter active in the feeding site only which may further enhance acceptance of transgenics. Moreover, the combination in one plant of different resistance genes with different resistance mechanisms will offer the opportunity to breed varieties with a more durable and broader resistance.

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