The *Sambucus nigra* type-2 ribosome-inactivating protein SNA-I′ exhibits in planta antiviral activity in transgenic tobacco

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Abstract Transgenic tobacco (Samsun NN) plants transformed with a cDNA clone encoding SNA-I′ from *Sambucus nigra* synthesize, and correctly process and assemble, a fully active type-2 ribosome-inactivating protein. Expression of SNA-I′ under the control of the 35S cauliflower mosaic virus promoter enhances the plant's resistance against infection with tobacco mosaic virus. In contrast to type-1 ribosome-inactivating proteins, the expression of SNA-I′ does not affect the growth and fertility of the transgenic plants and is not accompanied by an increased expression of pathogenesis-related proteins indicating that its antiviral activity most probably differs from that of pokeweed antiviral protein.

Key words: Antiviral activity; Ribosome-inactivating protein; Transgenic tobacco; *Sambucus nigra*

1. Introduction

RNA *N*-glycosylases (EC 3.2.2.22), also called 'ribosome-inactivating proteins' (RIPs), are an extended and heterogeneous family of plant proteins [1–3]. Most RIPs consist of an *N*-glycosylase domain of approximately 30 kDa. These RIPs are usually referred to as type-1 RIPs [3]. Other RIPs are chimeric proteins consisting of an N-terminal *N*-glycosylase domain tandemly arrayed to an unrelated C-terminal domain corresponding to either a lectin (as in type-2 RIPs) [3] or a protein with yet unidentified activity/function (as in the type-3 RIP JIP60) [4].

Though the function of RIPs is still not fully understood it has been suggested that they are involved in plant defense [3]. Since many type-1 RIPs are potent antiviral proteins in vitro and experiments with transgenic plants demonstrated that the type-1 RIPs from *Phytolacca americana* [5–8], *Phytolacca insularis* [9], *Trichosanthes kirilowii* [10] and *Dianthus caryophyllus* [11] act as antiviral proteins in planta there is an increasing belief that type-1 RIPs protect the plant against viruses. In contrast to type-1 RIPs, type-2 RIPs are not commonly considered antiviral proteins because their protective effect against plant viruses is poorly documented. Though clear indications for in vitro antiviral activity have been reported for abrin, ricin and moddecin [12] and for a type-2 RIP from *Eranthis hyemalis* [13], no evidence has been presented that any type-2 RIP exhibits in planta antiviral activity.

To corroborate the in planta antiviral activity of type-2 RIPs a type-2 RIP from *Sambucus nigra* (elderberry) (called *S. nigra* agglutinin I′ or SNA-I′) [14] was expressed in *Nicotiana tabacum* Samsun NN. Expression of SNA-I′ did not affect the phenotype and fertility of the transgenic lines but clearly enhanced their resistance against infection with tobacco mosaic virus (TMV) without an apparent increase of the expression of pathogenesis-related (PR) proteins. Our results not only demonstrate that a type-2 RIP exhibits in planta antiviral activity but also indicate that its mode of action may differ from that of the type-1 RIP pokeweed antiviral protein (PAP).

2. Materials and methods

2.1. Plasmid construction

Standard cloning techniques [15] were used to construct the plasmid for expression of SNA-I′. The complete coding sequence of SNA-I′ was amplified by PCR using the cDNA clone LECSNA-I′ [14] as a template with the primers 5′-ATGAAAGTGGTAGCAACAATATTA-3′ (5′ primer containing *Sac*I site for cloning) and 5′-CTATGCTGGATGGGTGCTAGCTAT-3′ (3′ primer with added *Nsi*I site). The restriction cassette (1.8 kb) was subcloned into the *Sac*I and *Nsi*I sites of the plasmid pCR2.1-TOPO (TOPO TA Cloning Kit, Invitrogen, CA, USA). After confirmation of the sequence by the dideoxy method [16], the plasmid was digested with *Sac*I and *Nsi*I and the insert cloned into the plant transformation vector pGB19 which was constructed by transfer of the *Eco*RI–*Hin*dIII fragment of the plasmid pFF19 [17] into pGPTV-BAR [18]. The resulting plasmid pGB19-SNA-I′ contained the SNA-I′ transgene under the control of the 35S promoter from cauliflower mosaic virus and the selectable marker phosphinothricin acetyltransferase (*bar*) under the control of the nopaline synthase promoter.

2.2. Transformation of tobacco

Agrobacterium tumefaciens GV3101 was transformed with the plasmid pGB19-SNA-I′ by electroporation and used for transformation of tobacco (Samsun NN) leaf discs [19]. Shoots were selected on Murashige and Skoog medium [20] with 0.1 mg/l α-naphthalene acetic acid, 1 mg/l 6-benzylaminopurine, 100 mg/l timentin, 100 mg/l cefotaxime, 100 mg/l carbenicillin and 5 mg/l phosphinothricin. Resistant shoots were transferred to Murashige and Skoog medium with 0.1 mg/l α-naphthalene acetic acid, 100 mg/l timentin, 100 mg/l cefotaxime, 100 mg/l carbenicillin and 5 mg/l phosphinothricin for rooting.

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Abbreviations: LECSNA-I′, cDNA encoding SNA-I′; PAP, pokeweed antiviral protein; PR, pathogenesis-related; RIP, ribosome-inactivating protein; SNA, *Sambucus nigra* agglutinin; TMV, tobacco mosaic virus

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2.3. RNA isolation and Northern blot analysis

RNA was prepared from tobacco leaves as described by Eggermont et al. [21], dissolved in RNase-free water and quantitated spectrophotometrically. Approximately 30 μg of total RNA was denatured in glyoxal and dimethylsulfoxide according to Sambrook et al. [15], and separated in a 1.2% (w/v) agarose gel. Following electrophoresis the RNA was transferred to Immobilon N membranes (Millipore, Bedford, MA, USA) and the blot hybridized [22] using a random-primer-labelled cDNA insert or a specific oligonucleotide probe for SNA-I′. Alternatively the blot was hybridized with probes encoding tobacco PR-1 and PR-3 proteins, and a wound-inducible protein kinase.

2.4. Isolation of SNA-I′ and recombinant SNA-I′

SNA-I′ was isolated from elderberry bark as described previously [14]. Recombinant SNA-I′ (rSNA-I′) was purified from tobacco leaves using the same procedure based on affinity chromatography on immobilized fetuin. The affinity-purified lectin was dialyzed against appropriate buffers and stored at −20°C until use.

2.5. Analytical methods

Crude extracts and purified proteins were analyzed by SDS–PAGE using 15% (w/v) acrylamide gradient gels as described by Laemmli [23]. Analytical gel filtration was performed on a Pharmacia Superose 12 column (Amersham Pharmacia Biotech, Uppsala, Sweden) using 0.1 M Tris–HCl (pH 8.7) containing 0.2 M NaCl and 0.2 M galactose as running buffer. The elderberry bark type-2 RIPs SNA-I (240 kDa) [24], SNA-V (120 kDa) [25] and SNLRP (60 kDa) [26] were used as molecular mass markers.

Protein concentration was determined with the Bradford assay [27] using SNA-I as a standard.

2.6. Agglutination and RNA N-glycosylase activity assays

Agglutination assays were done in 96 U-welled microtiter plates in a final volume of 50 μl containing 40 μl of a 1% suspension of trypsinized rabbit erythrocytes and 10 μl of extracts or lectin solutions. Agglutination was assessed visually after 1 h at room temperature.

The RNA *N*-glycosylase activity of SNA-I′ and rSNA-I′ was estimated using the method of Endo and Tsurugi [28] with some minor modifications. Ribosomes were incubated with purified RIPs in 25 mM Tris–HCl (pH 7.4) containing 25 mM KCl and 5 mM $MgCl_2$ at the appropriate temperature (37°C and 25°C for rabbit reticulocyte lysate and wheat germ ribosomes, respectively). After incubation total ribosomal RNA was extracted, dissolved in distilled H_2O and divided into two parts. One part was used as a control and the other was treated with a freshly prepared 1.0 M solution of acidic aniline (pH 4.5) at room temperature in the dark for 10 min to cleave the so-called Endo fragment. The RNA was analyzed in a 1.2% agarose–formamide gel. Gels were stained with 0.5 mg/ml ethidium bromide and photographed on a UV transilluminator.

2.7. Protein extraction and Western blot analysis

Samples (200 mg) of tobacco leaves were homogenized in 1 ml of 50 mM acetic acid using a Fastprep system and centrifuged at $13\,000 \times g$ for 5 min. Aliquots (200 μl) of the cleared extracts were lyophilized and dissolved in 20 μl of loading buffer (0.1 M Tris–HCl pH 7.8, 4% SDS, 10% glycerol containing 0.1% bromophenol blue and 1% β-mercaptoethanol). After electrophoresis, proteins were transferred to an Immobilon P membrane (Millipore, Bedford, MA, USA) using a semi-dry blotting system. Immunodetection was carried out as described by Desmyter et al. [29] using an affinity-purified polyclonal rabbit antibody raised against SNA-I from elderberry bark as the primary antibody.

2.8. Bioassay with TMV

Seeds of transgenic tobacco were sterilized by successive soaking in 70% ethanol and a solution of 5% NaOCl containing 0.05% Tween 20 before selection on MS medium containing 5 mg/l phosphinothricin. Seedlings with a healthy phenotype in the two-leaf stage were transferred to soil. In a later stage, the plants were checked for the expression of the lectin by a simple agglutination test on a small leaf sample. Only plants giving a strong agglutination activity were used for the experiments. When the selected plants reached the six-leaf stage the upper two fully expanded leaves were mechanically infected with TMV (strain TMV *vulgare*) by rubbing the virus suspension in 100 mM phosphate buffer (pH 7.2) containing 2% polyvinylpyrrolidone in the presence of carborundum powder on the leaves. Inoculated plants were kept in a greenhouse (16 h light, 55% humidity, 20–21°C day and 18–19°C night temperature) and after 4 days the number of local lesions on the infected leaves was counted. The size of the lesions (10 per plant) was measured under a microscope 7 days post-infection. Data obtained from each experiment were analyzed separately for statistical significance using SAS software [30].

3. Results

3.1. Selection and characterization of transgenic tobacco plants

Twelve independent phosphinothricin-resistant tobacco lines were regenerated from leaf discs transformed with *A. tumefaciens* harboring the vector pGB19-SNA-I′. Seven lines (designated 26101–26107) yielded a fragment with an expected size of 1.8 kb after PCR amplification of genomic DNA using specific primers for the complete coding sequence of LECSNA-I′ (data not shown). Northern blot analysis using a probe specific for SNA-I′ yielded clear hybridization signals for five out of seven putative transformants (Fig. 1A) whereas RNA from untransformed tobacco yielded no signal after hybridization with the same probe. Western blot analysis of crude leaf extracts confirmed that these five lines express polypeptides of approximately 35 kDa that react with antibodies against SNA-I (Fig. 1B) and are absent from untransformed tobacco plants. Calculations based on comparative agglutination assays (using purified SNA-I′ as a standard) indicated that the expression level of SNA-I′ in the transgenic plants varied between 0.25 and 2.5 μg/m protein. All tobacco plants expressing SNA-I′ showed a normal phenotype.

3.2. Purification and characterization of recombinant SNA-I′

rSNA-I′ was purified from leaves of transgenic tobacco plants. The overall yield was approximately 7.5 mg/kg leaves (fresh weight). SDS–PAGE under non-reducing conditions yielded a single polypeptide chain of approximately 65 kDa for both rSNA-I′ and SNA-I′. Under reducing conditions both type-2 RIPs yielded two polypeptides of approximately 35 kDa (results not shown). Native SNA-I′ and rSNA-I′ eluted at exactly the same position upon gel filtration on a Superose 12 column (results not shown) indicating that both

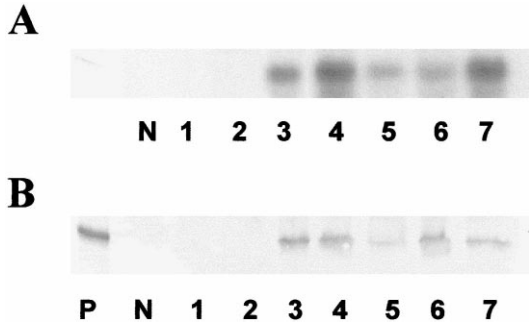

Fig. 1. A: Northern blot analysis of transformed tobacco lines. The blot was hybridized using a random-primer-labelled oligonucleotide probe specific for SNA-I′. B: Western blot analysis of transformed tobacco lines. Specific antibodies were used for the detection of SNA-I′ after blotting of the proteins. Samples were loaded as follows: lane P, pure SNA-I′ from elderberry; lane N, untransformed tobacco plant; lanes 1–7, transformed tobacco lines 26101, 26102, 26103, 26104, 26105, 26106 and 26017, respectively. According to densitometric analyses the expression level of SNA-I′ in transgenic lines 26103, 26104, 26105, 26106 and 26017 corresponds to 2.52, 1.64, 0.66, 1.41 and 1.11 μg/mg total soluble protein, respectively.

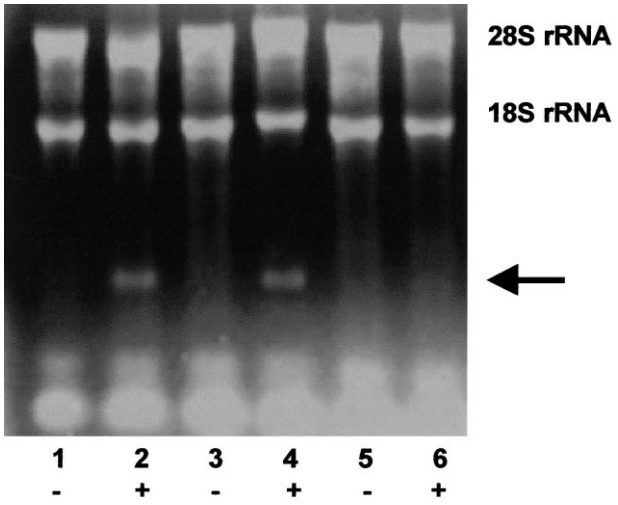

Fig. 2. RNA *N*-glycosylase activity of native and recombinant SNA-I′ towards rabbit reticulocyte lysate ribosomes. RNA bands were visualized by ethidium bromide staining. (−) and (+) indicate no treatment and aniline treatment, respectively. The arrow indicates the position of the Endo fragment released from the rRNA. Samples were loaded as follows: lanes 1–2, 1 mM native SNA-I′; lanes 3–4, 1 mM rSNA-I′; lanes 5–6, crude protein extract of untransformed tobacco.

the native and the recombinant protein are dimeric type-2 RIP with the same $[A\text{-}s\text{-}s\text{-}B]_2$ structure. According to these data the cells of transgenic tobacco plants synthesize and correctly process and assemble SNA-I′.

RNA *N*-glycosylase assays demonstrated that rSNA-I′ acts as an RNA *N*-glycosylase on rabbit reticulocyte but not on wheat germ ribosomes (Fig. 2). Parallel experiments with SNA-I′ yielded identical results. rSNA-I′ and SNA-I′ were equally active the minimal concentration required for RNA *N*-glycosylase activity being 50 pM.

Semi-quantitative agglutination assays indicated that rSNA-I′ and SNA-I′ are equally active, the minimal concentration required for the agglutination of trypsin-treated rabbit erythrocytes being 40 μg/ml. Moreover, rSNA-I′ and SNA-I′ are also equally sensitive towards lactose (20 mM) and fetuin

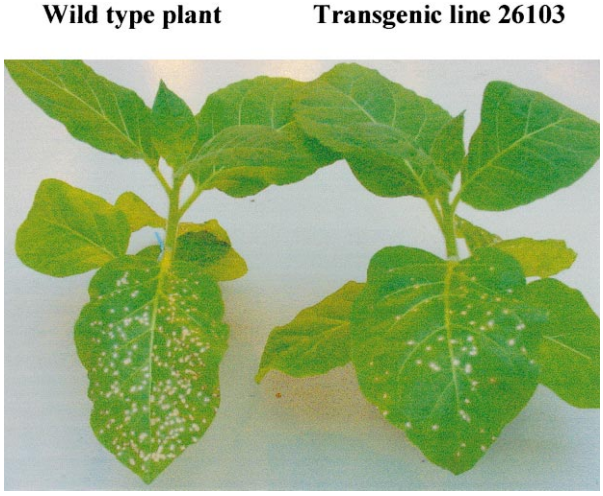

Fig. 3. Development of local lesions on the leaves of untransformed tobacco plant and transgenic line 26103 upon infection with TMV. The picture was taken 4 days post-infection.

(125 μg/ml), suggesting that the B-chain of the recombinant lectin exhibits the same carbohydrate-binding specificity as SNA-I′.

3.3. *Expression of SNA-I′ enhances the resistance of tobacco plants against infection with TMV without increasing the expression of PR proteins*

Transgenic tobacco plants expressing the SNA-I′ were mechanically infected with TMV and the development of symptoms of viral infection compared to those occurring in untransformed control plants. Both the number of lesions and the lesion size was reduced in the transgenic lines (Table 1 and Fig. 3), which clearly indicates that the expression of SNA-I′ enhances the resistance of transgenic tobacco plants against infection with TMV.

A comparison of the levels of transcripts encoding PR-1 and PR-3 proteins, and wound-inducible protein kinase by Northern blot analysis did not show any detectable difference between the transgenic lines and untransformed plants neither in young nor in old leaves (results not shown).

4. Discussion

During the last decade conclusive evidence was reported that the expression of the type-1 RIPs from *P. americana* [5–8] and *T. kirilowii* [10] protects transgenic tobacco and potato plants against infection with various plant viruses. To corroborate whether type-2 RIPs also increase the plant's resistance against viruses, the gene encoding SNA-I′ from elderberry was transferred into tobacco and the resulting transgenic plants tested for resistance against TMV infection. Transgenic tobacco plants expressing SNA-I′ clearly exhibit an increased resistance against infection with TMV. These results demonstrate for the first time that a type-2 RIP exerts antiviral activity in planta, which strongly suggests that not only type-1 RIPs but also type-2 RIPs may be involved in plant defense. In contrast to many type-1 RIPs, the ectopic expression of SNA-I′ causes no visible phenotype indicating that this elderberry type-2 RIP exerts no cytotoxic effects in planta. This obvious absence of a phenotype strikingly contrasts with the detrimental effects of ectopically expressed type-1 and type-3 RIP. For example, tobacco plants expressing PAP at a concentration of >10 ng/mg protein showed a stunted, mottled phenotype and plants with the highest expression level were sterile [5]. Expression of the type-3 RIP JIP60 in transgenic tobacco under the control of a constitutive

Table 1
Effect of the ectopic expression of SNA-I′ on the infection of transgenic tobacco plants with TMV

Plant line	Number of lesions per leaf[a]		Lesion size[b]	
	total number	% of control	mm	% of control
Wild type	102	100	2.15	100
26103	42	41**	1.22	57**
26104	49	48**	1.48	69*
26106	84	82*	1.63	75*
26107	84	82*	1.66	77*

*$P<0.05$, **$P<0.01$, significantly different from wild type.
[a]The number of local lesions on the infected leaves was counted after 4 days. Individual values for lesion numbers are the means of three replicates.
[b]The size of the lesions (six per plant) was measured under a microscope 7 days post-infection.

promoter also resulted in reduced growth, shorter internodes, lanceolate leaves, reduced root development and premature leaf senescence [31]. The obvious absence of a phenotype in the tobacco plants expressing SNA-I′ cannot be ascribed to a low expression level of the type-2 RIP. On the contrary, the expression level of SNA-I′ in our experiments (up to 2.5 μg/mg protein) exceeds the expression levels of PAP (10 ng/mg protein) [5] and PAP II (150 ng/mg protein) [8] that caused a phenotype in tobacco by a factor 250 and 17, respectively.

In contrast to wild-type PAP and various PAP mutants the ectopic expression of SNA-I′ is not accompanied by enhanced expression of wound-induced protein kinase and PR-1 and PR-3 proteins in transgenic tobacco [6,7,32]. This observation is important because it indicates that the proposed mode of action of type-1 RIPs, which according to the latest ideas relies on a salicylic acid-independent signal transduction pathway reminiscent of the wounding response [3,7,32], cannot simply be extrapolated to SNA-I′. It is questionable, therefore, whether the observed in planta antiviral activity of the elderberry type-2 RIP results from the same activation of an innate plant defense system as that proposed for PAP. This may also explain why the in planta protective effect of SNA-I′ is apparently lower than that of PAP and other type-1 RIPs.

Analysis of SNA-I′ isolated from transgenic tobacco demonstrated that the recombinant protein exhibits virtually the same enzymatic and carbohydrate-binding activities as native SNA-I′ from elderberry bark, indicating that the tobacco cells synthesize and correctly process and assemble this elderberry type-2 RIP. Hitherto, only the monomeric type-2 RIP ricin was expressed in tobacco plants [33]. Our results demonstrate for the first time that transgenic tobacco is capable of expressing not only a simple monomeric type-2 RIP like ricin but also an oligomeric type-2 RIP with a complex structure.

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