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Carbohydrate-binding activity of the type-2 ribosome-inactivating protein SNA-I from elderberry (*Sambucus nigra*) is a determining factor for its insecticidal activity

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ABSTRACT

In recent years, different classes of proteins have been reported to promote toxic effects when ingested. Type-2 ribosome-inactivating proteins (RIPs) are a group of chimeric proteins built up of an A-chain with RNA *N*-glycosidase activity and a B-chain with lectin activity. These proteins are thought to play a role in plant protection. *Sambucus nigra* agglutinin I (SNA-I) is a type-2 RIP, isolated from the bark of elderberry (*S. nigra* L.). This study demonstrated the insecticidal potency of SNA-I on two Hemipteran insect species using two different methods. An artificial diet supplemented with different concentrations of the purified RIP reduced survival and fecundity of pea aphids *Acyrthosiphon pisum*. In addition, feeding of tobacco aphids, *Myzus nicotianae*, on leaves from transfected plants constitutively expressing SNA-I, resulted in a delayed development and reduced adult survival and also the fertility parameters of the surviving aphids were reduced, suggesting that a population of aphids would build up significantly slower on plants expressing SNA-I. Finally, a series of experiments with transgenic lines in which a mutant RIP was expressed, revealed that the carbohydrate-binding activity of SNA-I is necessary for its insecticidal activity. In a first set of mutants, the B-chain was mutated at one position (Asp231ΔGlu), and in the second set both carbohydrate-binding sites were mutated (Asn48ΔSer and Asp231ΔGlu). Mutation of one carbohydrate-binding site strongly reduced the insecticidal activity of SNA-I, whereas mutation of both lectin sites (almost) completely abolished the SNA-I effect on tobacco aphids.

1. Introduction

Aphids represent a very important group of pest insects, causing both direct damage to plants and indirect damage by transmitting important viruses that can devastate agricultural crops. Intensive use of insecticides has stimulated outbreaks of other pests and also appearance of resistance and cross resistance in many cases (Devonshire et al., 1998; Foster et al., 2002).

In recent years, a number of different classes of proteins have been reported to promote toxic effects when ingested by plant sucking Hemiptera (Cooper and Goggin, 2005; Dutta et al., 2005). One of the plant proteins that may be involved in plant protection are ribosome-inactivating proteins (RIPs) which are classically divided into three groups: Type-1 RIPs consist of a single chain with enzymatic activity. Type-2 RIPs are a group of chimeric proteins built up of an A-chain and a B-chain that are linked by a disulfide bond. The A-chain or RNA *N*-glycosylase domain (EC 3.2.2.22) binds to one specific adenosine of the large ribosomal RNA and catalyses the endohydrolysis of the *N*-glycosidic bond, whereas the B-chain comprises a typical lectin domain (Van Damme et al., 2001; Stirpe, 2004). This B-chain contains at least two binding sites that enable the protein to interact with carbohydrates on the cell surface receptor. Type-3 RIPs are proteins composed of an *N*-terminal RNA *N*-glycosidase domain and a *C*-terminal domain, the function of which has not been clarified (Peumans et al., 2001). It is clear now that RIPs do not only deadenylate rRNA but can remove adenine residues from various polynucleotides other than rRNA (Barbieri et al., 1997).

Some RIPs have been proposed to play a role as a storage protein (Van Damme et al., 2001; Liu et al., 2002). In addition, RIPs have also been suggested to be involved in plant defence against viruses, fungi and insects. Several studies have demonstrated the insecticidal activity of RIPs on different insect orders such as Coleoptera (Gatehouse et al., 1990; Kumar et al., 1993), Lepidoptera (Dowd et al., 1998, 2003, 2006; Zhou et al., 2000; Wei et al.,

Abbreviations: RCA, *Ricinus communis* agglutinin; RIP, ribosome-inactivating protein; SNA-I, *Sambucus nigra* agglutinin I.

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2004), Diptera (Zhou et al., 2000). It should be mentioned that also non-RIP lectins have been reported to have insecticidal properties (Van Damme, 2008). Particularly mannose-binding lectins and legume lectins have been shown to possess insecticidal activity (Fitches et al., 2001a,b; Sauvion et al., 2004; Wang et al., 2005).

Sambucus nigra agglutinin I (SNA-I) is a type-2 RIP, isolated from elderberry (*S. nigra* L.) bark, with an exclusive specificity toward the Neu5Ac(α2,6)Gal/GalNAc disaccharide and a unique molecular structure typified by the occurrence of a disulfide bridge between the B-chains of two adjacent protomers (Van Damme et al., 1996; Chen et al., 2002a). SDS–PAGE of reduced and iodoacetylated SNA-I has demonstrated that it is composed of two different polypeptides, namely an A-chain of 33 kDa and a B-chain of 35 kDa (Van Damme et al., 1996). Molecular cloning and sequence analysis demonstrated that the B-chain consists of six randomly arrayed subdomains (1α, 1β, 1γ, 2α, 2β, 2γ) of approximately 40 amino acid residues. Although all the subdomains share high sequence similarity and have a similar structure, presumably only subdomains 1α and 2γ possess a functional carbohydrate-binding site (Van Damme et al., 2001).

This study aimed at investigating and defining the insecticidal potency of SNA-I, as well as the importance of its carbohydrate-binding activity for toxicity. Therefore, the insecticidal activity of SNA-I was tested on two Hemipteran insect species using two different methods. To check the intrinsic insecticidal activity of the type-2 RIP SNA-I against aphids the pure RIP was added to an artificial diet that was optimized for investigations with pea aphids (*Acyrthosiphon pisum*). In addition, transgenic plants constitutively expressing SNA-I were used in bioassays with tobacco aphids (*Myzus nicotianae*). This experiment allowed evaluating the practical application of the SNA-I as an insecticidal protein recombinantly expressed in a host plant.

In order to demonstrate the importance of the carbohydrate-binding activity of SNA-I for insecticidal activity, the assays with transgenic plants were also repeated using transgenic lines in which a mutant RIP was expressed. In a first set of mutants (SNA-I 103M1 and SNA-I 107M1) the B-chain was mutated at one position: namely Asp231ΔGlu in site 2γ. In a second set of plants (SNA-I 109M2 and SNA-I 112M2) both carbohydrate-binding sites were mutated: namely Asn48ΔSer in site 1α and Asp231ΔGlu in site 2γ. Previously it was shown that the double mutant RIP does no longer possess carbohydrate-binding activity, whereas the lectin activity in the single mutant is strongly reduced (Chen et al., 2002b).

2. Results

2.1. Bioassays with artificial diet supplemented with purified SNA-I and pea aphids, A. pisum

2.1.1. Effect on survival of nymphs

Different concentrations of purified SNA-I (1, 5, 10, 25, 50, 100, 200, 400, 800, 1000 and 1200 μg/ml) were added to an artificial diet and tested for acute insecticidal activity. As shown in Fig. 1, the dose response effect of SNA-I on the survival of neonate nymphs of *A. pisum* after 3 days, demonstrated that doses of >100 μg/ml of SNA-I were clearly toxic for the pea aphids. Using sigmoid curve analysis an LC_{50} value of 374 μg/ml was calculated.

2.1.2. Effect on survival and fecundity of adults

From Fig. 1 it is clear that concentrations of ⩽100 μg/ml of SNA-I showed a poor acute toxic effect on survival of *A. pisum*, and therefore the effect of these doses was tested for a period of 18 days against the survival from the first instar nymphal until the adult stage, and against the fecundity of the surviving pea aphid (approximately 8 days of reproduction) (Table 1). On day 18, adult mortality scored after Abbott correction was 45–57% in aphids treated with SNA-I at 100, 50 and 25 μg/ml, and 15% with 5 μg/ml. A dose of 100 μg/ml SNA-I significantly reduced the mean daily offspring and total fecundity by 69% and 70%, respectively, as compared to the control aphids. With a lower dose of 50 μg/ml SNA-I, 42% and 43% reductions were observed for mean daily offspring and total fecundity, respectively.

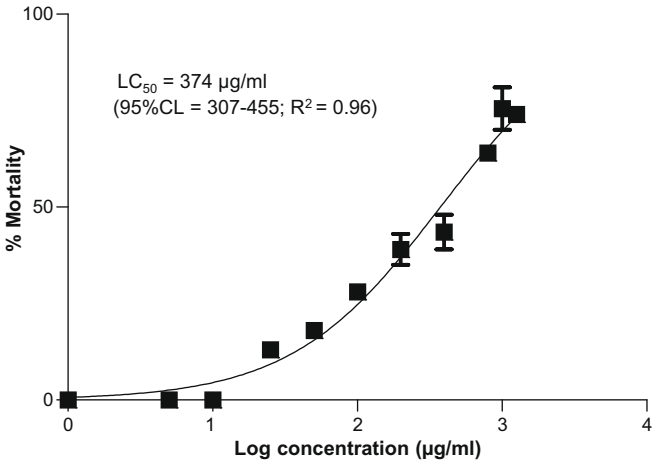

Fig. 1. Dose response curve of mortality by SNA-I in *Acyrthosiphon pisum* after three days. Nymphal mortality percentages are based on two repeated experiments, each consisting of four groups of 15 nymphs each; a total of 120 aphids was tested per concentration. Data are corrected for mortality in the controls (0–20%) using Abbott's formula.

2.2. Bioassays with transgenic lines expressing SNA-I and tobacco aphids, M. nicotianae

2.2.1. Effect on survival of nymphs and adults

Transgenic tobacco lines constitutively expressing the native SNA-I (SNA-I 101 and SNA-I 105) or one of its mutant forms (SNA-I 103M1, SNA-I 107M1, SNA-I 109M2 and SNA-I 112M2) were used to test the effect of the RIP on survival of the tobacco aphid *M. nicotianae*. It should be noted that none of the transgenic tobacco lines showed any phenotypical differences when compared to control, untransformed tobacco plants. Semi-quantitative determination of the agglutination activity in these transgenic lines confirmed that only the plants expressing the native RIP showed clear agglutination activity. For tobacco lines SNA-I 101 and SNA-I 105, respective lectin concentrations of 17 ± 7 and 20 ± 4 μg/g FW were calculated. The plants expressing the single mutant RIP showed a strongly reduced agglutination activity. For SNA-I 103M1, the total leaf lectin content was calculated 4 ± 0.3 μg/g FW, and in the case of SNA-I 107M1, lectin activity

Table 1
Effect of different concentrations of SNA-I on survival and fecundity of *Acyrthosiphon pisum* when supplemented into the artificial diet for a period of 18 days

SNA-I dose (μg/ml)	Total fecundity (larvae/adult)[a]	Mean daily offspring[b]
100	1.7 ± 0.1*	0.19 ± 0.06[b]
50	3.2 ± 1.5	0.36 ± 0.10[ab]
25	4.4 ± 0.2	0.46 ± 0.08[ab]
5	4.8 ± 0.5	0.53 ± 0.08[ab]
Control	5.6 ± 0.6	0.62 ± 0.10[a]

[a] To separate means of total progeny, a non-parametric Mann–Whitney *U*-test was carried out ($Z = -2.12$; $p < 0.05$); data followed by an asterisk * are significantly lower than the control.

[b] To separate means (a, b) of daily offspring, ANOVA (two groups; $F = 3.72$; df = 40; $p < 0.05$) was followed by a *post-hoc* Tukey test ($p = 0.05$).

was barely detectable. Similar to control tobacco plants no agglutination activity was detected in the plants (SNA-I 109M2 and SNA-I 112M2) expressing the double mutant protein.

Per transgenic line, a total of 60 neonate nymphs (aged 0–24 h) was tested over a period of 35 days to determine the insecticidal effect on nymphs and adults. With nymphs, no effects on survival were observed (Fig. 2). But for adults, feeding on the transgenic lines SNA-I 101, SNA-I 105, SNA-I 103M1 and SNA-I 107M1 caused a significant reduction of their survival ($p < 0.001$) (Table 2). The transgenic lines SNA-I 109M2 ($p = 0.06$) and SNA-I 112M2 ($p = 0.68$) did not show an effect compared to the controls. As shown in Fig. 2, with the lines SNA-I 101, SNA-I 105 and SNA-I 103M1 survival reduction started 8–9 days after adult formation, and in SNA-I 107M1 after 9–10 days, whereas in the control and the double mutant lines this decline started only after 13–15 days. By day 30, the numbers of adult aphids on SNA-I 101, SNA-I 105, SNA-I 103M1 and SNA-I 107M1 were significantly ($p < 0.05$) reduced and yielded 20%, 0%, 31%, and 41%, respectively, compared to 62% in the controls. It is worth noting that all aphids were killed after 30, 31, and 32 days on the transgenic lines SNA-I 105 and SNA-I 101 and SNA-I 103M1, respectively, whereas at that moment still 35% of the aphids was alive on the control plants and 32% on the two double mutant lines.

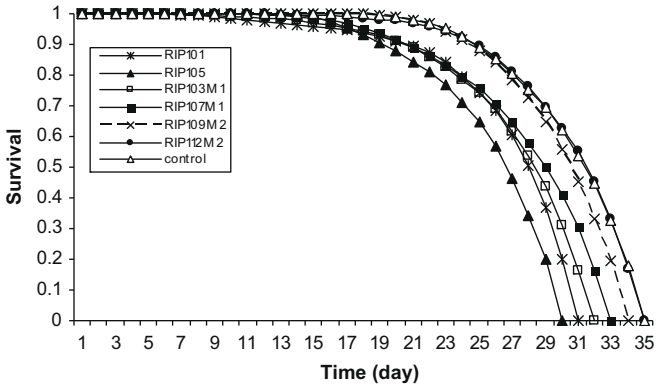

Fig. 2. Nymphal and adult survival of *Myzus nicotianae* on tobacco plants expressing native or mutant SNA-I compared to wild type tobacco plants (control).

2.2.2. Effect on life table parameters of M. nicotianae

Non-mutated and mutated (either one or two sites mutated) transgenic tobacco lines were used to follow the effect on fecundity and development of *M. nicotianae*. The mean duration of the prereproductive period, defined as the period of time from birth until onset of reproduction, was significantly reduced for aphids fed on the transgenic lines SNA-I 101, SNA-I 105 and SNA-I 103M1 as compared to the control ($p < 0.05$), whereas no effect was observed on SNA-I 107M1, SNA-I 109M2 and SNA-I 112M2 ($p > 0.05$) (Table 2).

Table 2 also demonstrates that the total fecundity in aphids fed on transgenic lines SNA-I 101, SNA-I 105 and SNA-I 103M1 was significantly reduced as compared to the control. Also the intrinsic rate of increase (r_m) was significantly lower in aphids on the non-mutated and one site mutated lines, and this in turn resulted in lower finite ratios of increase (λ). In contrast the two double mutant lines yielded a similar intrinsic rate and finite ratio of increase as the controls. When calculating the doubling time (DT), which is the number of days in which the aphid population can double in size, an increase of about 20% was observed for the transgenic lines SNA-I 101, SNA-I 105 and SNA-I 103M1 as compared to wild type plants (Table 2).

Over the period of reproductive performance of the aphids which is expressed in the mean total number of nymphs born per adult every single day for the entire reproductive period ($R_0 = \sum L_x M_x$) a significant reduction was assessed in aphids fed on transgenic lines SNA-I 101, SNA-I 105 and SNA-I 103M1. As shown in Fig. 3, the cumulative fecundity on the lines SNA-I 101, SNA-I 105, SNA-I 103M1 by day 35 was 23% and 21% and 18% lower than in the controls, respectively.

3. Discussion

Although the biochemical properties of the RIPs are well studied, their mechanism of action on insects is not fully understood. During the last two decades insecticidal activity was reported for a few RIPs. Gatehouse et al. (1990) have shown that RIPs from the seeds of dicotyledonous plants, such as ricin (type-2 RIP) from castor bean (*Ricinus communis*) and saporin (type-1 RIP) from soapwort (*Saponaria officinalis*), were toxic to beetles but not to caterpillars. The type-2 RIP cinnamomin from *Cinnamomum camphora* was shown to be toxic to bollworm and mosquito (Zhou et al., 2000; Wei et al., 2004). Dowd et al. (1998) reported the activity of a type-3 RIP from maize against both beetles and caterpillars.

Table 2
Life table parameters of *Myzus nicotianae* adults fed on different transgenic tobacco lines expressing the native SNA-I (SNA-I 101, SNA-I 105), single mutant (SNA-I 103M1 and SNA-I 107M1), or double mutant (SNA-I 109M2 and SNA-I 112M2), compared to control plants

Line	Total fecundity	Mean generation time (day)	Intrinsic rate of increase r_m[a]	Mean daily offspring	Doubling time DT (day)	Prereproductive time (day)	Finite rate of increase (λ)
SNA-I 101	51.6 ± 2.5^{b}	13.97 ± 0.05^{c}	0.258 ± 0.007^{c}	2.7 ± 0.1^{c}	2.70 ± 0.06^{c}	7.65 ± 0.19^{b}	1.30 ± 0.005^{cd}
SNA-I 105	49.6 ± 3.1^{b}	14.18 ± 0.02^{c}	0.262 ± 0.005^{c}	3.3 ± 0.1^{bc}	$2.69 \pm 0.0\ 5^{c}$	7.52 ± 0.11^{b}	1.30 ± 0.005^{cd}
SNA-I 103M1	53.1 ± 2.6^{b}	14.61 ± 0.05^{d}	0.257 ± 0.007^{c}	3.3 ± 0.2^{bc}	2.69 ± 0.07^{c}	7.63 ± 0.17^{b}	1.29 ± 0.004^{d}
SNA-I 107M1	59.7 ± 2.9^{ab}	14.05 ± 0.12^{c}	0.280 ± 0.005^{bc}	3.1 ± 0.1^{bc}	2.50 ± 0.04^{bc}	7.29 ± 0.19^{ab}	1.32 ± 0.004^{bc}
SNA-I 109M2	61.2 ± 2.4^{ab}	12.79 ± 0.12^{a}	0.313 ± 0.003^{a}	3.8 ± 0.1^{ab}	2.35 ± 0.07^{ab}	6.92 ± 0.12^{a}	1.35 ± 0.008^{a}
SNA-I 112M2	66.5 ± 3.5^{a}	13.46 ± 0.10^{b}	0.289 ± 0.008^{ab}	3.4 ± 0.1^{bc}	2.43 ± 0.05^{ab}	6.83 ± 0.15^{a}	1.33 ± 0.007^{ab}
Control	65.4 ± 2.0^{a}	12.81 ± 0.10^{a}	0.313 ± 0.005^{a}	4.4 ± 0.3^{a}	2.24 ± 0.04^{a}	6.96 ± 0.09^{a}	1.36 ± 0.005^{a}

Data are presented as means ± SE based on two repeated experiments, each consisting of 15 aphids; per transgenic lines 30 aphids have been tested. For each column, ANOVA (two groups for "total fecundity", $F = 6.14$, df = 6,88; $p < 0.001$; four groups for "mean generation time", $F = 104.44$, df = 6,209; $p < 0.001$; three groups for "intrinsic rate of increase", $F = 14.89$, df = 6,175; $p < 0.001$; three groups for "mean daily offspring", $F = 9.44$, df = 6,149; $p < 0.001$; three groups for "doubling time", $F = 9.55$, df = 6,172; $p < 0.001$; four groups for "finite rate of increase", $F = 19.20$, df = 6,187, $p < 0.001$); two groups for "prereproductive time", $F = 5.05$, df = 6,168; $p < 0.05$) was followed by a *post-hoc* Tukey test to separate means (a, b, c, d) at $p = 0.05$.

[a] Jackknife estimator (SD_{jack}) was used as standard error.

More recently, they also showed enhanced resistance of tobacco plants expressing an activated form of maize RIP (type-3 RIP) against *Helicoverpa zea*, as well as significant feeding reduction and larval mortality in the Lepidopteran *H. zea* and the Coleopteran *Lasioderma serricorne* after feeding on transgenic tobacco plants expressing the maize RIP (Dowd et al., 2003, 2006). All these results obtained with transgenic plants expressing RIP or purified RIP incorporated into artificial diet, confirm the insecticidal activity of RIPs on Lepidoptera and Coleoptera, but until now there is no report on insecticidal activity of RIPs on Hemipterans.

The results of this investigation showed the insecticidal activity of the type-2 RIP SNA-I on *A. pisum* and *M. nicotianae* using two independent assays. Incorporation of different concentrations of the purified SNA-I in an artificial diet revealed an adverse effect on *A. pisum* survival. Concentrations of 100 μg/ml SNA-I or higher revealed a clear toxic effect on the pea aphid, the LC_{50} being 374 μg/ml. This finding underlines the first objective of this study, namely to confirm the intrinsic insecticidal activity of the type-2 RIP SNA-I against aphids. In this bioassay with artificial diet, we could use *A. pisum* as an aphid species, representing the family of Aphididea in the order of Hemiptera; *M. nicotianae* aphids also belonging to the Aphididae could not be maintained on the artificial diet.

In addition, it was of interest that lower concentrations of SNA-I (<100 μg/ml) also showed insecticidal effects with a clear reduction in mean daily offspring and total fecundity, indicating that sublethal doses of SNA-I can also affect the progeny of pea aphids. The results of this artificial diet assay with a reduced aphid survival, fitness and reproduction were confirmed by bioassays with transgenic plants overexpressing SNA-I. Feeding of *M. nicotianae* on transgenic plants resulted in a clear delay in development and a reduction in adult survival. In addition, the fertility parameters of the aphids were also reduced, suggesting a much slower population build up on transgenic plants expressing SNA-I compared to control tobacco plants. As a consequence, these findings suggest that SNA-I is a suitable candidate as an insect resistance protein for the control of aphids through a transgenic approach. It can be remarked that the *in planta* experiments were performed with tobacco aphids *M. nicotianae* while the artificial diet assays were done with pea aphids *A. pisum*. In addition there were also differences in the experimental design of the two types of experiments. In the experiments conducted with artificial diet and *A. pisum* total mortality was scored after 18 days, while in the experiments with transgenic plants total mortality was scored after 30 days in which the natural mortality was also included. Therefore, a direct correlation between the *in planta* results and the artificial diet data is not possible. However, since *A. pisum* and *M. nicotianae* belong both to the same family of Aphididea in the order of Hemiptera, we believe that the insecticidal activity scores with the artificial diet experiments with pure SNA-I are already very good indicators of an insecticidal activity with transgenic plants constitutively expressing SNA-I. From own previous experiments (unpublished), we see that the artificial diet assay allows easy screening under controlled conditions for insecticidal activities of proteins and other toxicants against aphids, and that these data are a good indication for *in planta* activity. As we optimized the technique with artificial diet, it is suitable for short- and long-term studies involving the effects of proteins and other toxicants on aphids. In the future, it may also

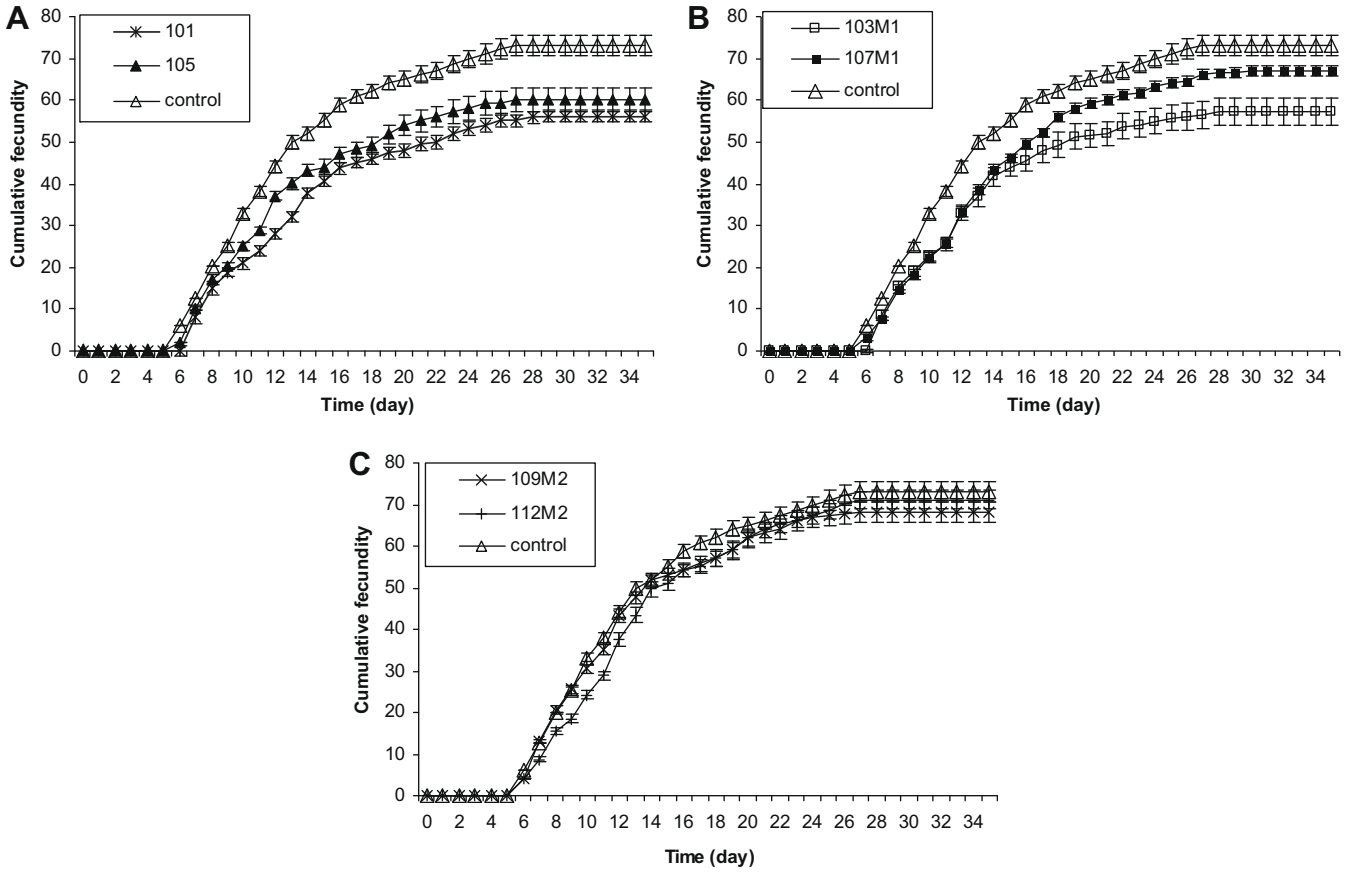

Fig. 3. Cumulative nymph production of surviving *Myzus nicotianae* adults fed on tobacco plants expressing native or mutant SNA-I compared to wild type tobacco plants (control).

be used to study the effects of growth factors, hormones and special nutrients or conditions required for aphid growth, and possibly also other sucking insects.

Recently, the insecticidal activity of SNA-I′ was described on two major pest insects (*M. nicotianae* and *Spodoptera exigua*) (Shahidi-Noghabi et al., in press). SNA-I′ is another elderberry lectin that is closely related to SNA-I, but it differs in its molecular structure. Compared to SNA-I, SNA-I′ lacks the extra cysteine residue, which is involved in the disulfide bridge formation between the B-chains of SNA-I. A comparison of the insecticidal effect of transgenic lines, expressing SNA-I and SNA-I′ at a similar level of 17-21 μg/g FW, revealed that the biological parameters of the challenged *M. nicotianae* aphids were negatively affected to a similar extend. For instance, the generation time, population doubling time and prereproductive period were increased by 9–17%, 21–22% and 11–12%, respectively, and the total fecundity was reduced by 21–24%. Consequently, the intrinsic rate of increase and finite rate of increase were reduced by 18–19% and 4–7%. Therefore, it can be concluded that the biological activity of these two lectins SNA-I and SNA-I′ is similar.

To further understand the mechanism of action of SNA-I we used mutated lines in which one or two carbohydrate-binding sites in the B-chain were disrupted. A comparative analysis of the insecticidal activity of SNA-I and its mutant forms allowed to determine the importance of the carbohydrate-binding activity of the RIP for its insecticidal activity. Bioassays with the different transgenic tobacco plants revealed a more strongly reduced adult survival and delayed aphid development to adulthood for *M. nicotianae* fed on transgenic lines expressing native SNA-I as compared with aphids fed on control plants and plants expressing the mutant RIPs. Feeding on transgenic lines expressing SNA-I also significantly reduced the intrinsic rate of increase of the tobacco aphids, whereas no significant reduction was observed on the transgenic lines expressing the mutant RIPs nor on the control plants. A clear effect of SNA-I on the longevity of the aphids was observed, since the decline in survival of the aging aphid population occurred more rapidly in the presence of SNA-I. The plants expressing the single mutant RIPs showed a lower survival rate than the transgenic plants expressing the double mutant SNA-I. Similarly, fertility parameters, such as mean daily offspring, total fecundity and intrinsic rate of increase were reduced in transgenic lines expressing the single mutant SNA-I compared to the double mutant SNA-I. It is worthy to note that aphid development took considerably longer in the aphids fed on transgenic lines expressing native SNA-I and the single mutant compared to the control plants and the double mutant transgenic lines. Chen et al. (2002b) have shown before that the amino acid changes introduced into the carbohydrate-binding sites of SNA-I strongly reduced (in case of mutation of site 2γ) or even abolished (in case of mutation of both sites) the carbohydrate-binding activity of the protein but did not affect the RIP activity. Furthermore they showed that the mutation Asp231ΔGlu in the single mutant SNA-I reduces the affinity of the RIP B-chain for galactose and fetuin by more than 50%. Furthermore, the introduction of the second mutation Asn48ΔSer reduces the binding activity to less than 20% of the original activity. The results of our experiments with the mutant SNA-I revealed that abolishing one carbohydrate-binding site resulted in a reduced insecticidal activity compared to native SNA-I. Mutation of both carbohydrate-binding sites of SNA-I (almost) completely abolished the insecticidal effect of the RIP since the results of the double mutant strongly resembled the results obtained with the control tobacco plants.

As reported by Stirpe and Battelli (2006), the B-chain of most type-2 RIPs binds to galactosyl-terminated receptors on the surface of the cells, thus allowing entry of the A-chain inside the cell, where it inactivates ribosomes with consequent arrest of protein synthesis and death of the cell. Although sugar binding sites of the B-chain share a high degree of similarity in sequence and three-dimensional structures, their sugar binding specificities appear to differ and this variability might be important in the binding of the B-chains to the potential sugar-containing receptors on the cell surface.

Previously it was shown for ricin that a single point mutation (Lys40ΔMet or Asn46ΔGly in site 1, or Asn255ΔGly in site 2) in one of the galactose binding sites of the ricin B-chain resulted in a 20- to 40-fold lower cytotoxicity compared to the wild type ricin, whereas a double mutant RIP with mutations in the two carbohydrate-binding domains (double site mutant containing all three amino acid replacements) exhibited no detectable cytotoxicity (Newton et al., 1992). These results also confirm that carbohydrate-binding activity is crucial for cytotoxicity of ricin. Furthermore, Sphyris et al. (1995) have reported that both sites of the *R. communis* agglutinin (RCA) B-chain must be simultaneously modified in order to abolish lectin activity, indicating the presence of two independent, functional binding sites. The activity associated with the carbohydrate-binding site 2 of the RCA B-chain was abrogated by the conversion of Trp258 to Ser. Moreover, the domain 2 site appears responsible for a weak binding interaction of recombinant RCA B-chain with GalNAc, which was not observed with the native tetrameric RCA. It has also been described that the introduction of His at position 248 of the ricin B-chain severely disrupted but did not abolish completely GalNAc binding (Sphyris et al., 1995). Interestingly, de Sousa et al. (1999) reported the restoration of lectin activity in an inactive abrin (type-2 RIP) B-chain by substitution and mutation of the 2γ-subdomain. Recently, it has also been demonstrated that lower cytotoxicity of cinnamomin is due to some amino acid changes in its B-chain (compared to ricin) (Wang et al., 2006). It has been also proposed that the difference in toxicity of two type-2 RIPs cinnamomin and ricin to domestic silkworm larvae (*Bombyx mori*) is related to the properties of their B-chains and is not related to their A-chains (Liu et al., 2002; Wei et al., 2004).

In contrast to most type-2 RIPs that are specific for galactose and GalNAc, several elderberry RIPs recognize sialic acid (Van Damme et al., 2001). The replacement of amino acid residues Ser197, Ala233 and Gln234, in the *C*-terminal carbohydrate-binding site of *Sambucus sieboldiana* agglutinin (SSA) B-chain revealed their importance for binding to sialic acid in the Neu5Acα2,6 Gal/GalNAc sequence (Kaku et al., 2007). In addition, our results have shown that the single mutant SNA-I which shows a strongly reduced binding to sialic acid also revealed a strongly reduced insecticidal activity, and that the double mutant RIP that no longer binds sialic acid had lost its insecticidal almost completely. The latter data suggest that the activity of the B-chain is required for insecticidal activity of SNA-I.

4. Experimental

4.1. Plants

Tobacco (*Nicotiana tabacum* cv Samsun NN) plants have been transformed with the gene encoding SNA-I from *S. nigra* (Chen et al., 2002b). Transgenic tobacco lines expressing the native RIP as well as two mutant lines affected in one or two carbohydrate-binding sites were obtained as described previously. All RIP constructs are expressed using the 35S cauliflower mosaic virus promoter in order to obtain constitutive expression of the protein. Six independent transgenic tobacco lines (SNA-I 101, SNA-I 105, SNA-I 103M1, SNA-I 107M1, SNA-I 109M2, SNA-I 109M2) as well as wild type tobacco (*N. tabacum* cv Samsun NN) plants were grown in a growth chamber with 25 ± 2 °C, 16 h light: 8 h dark photoperiod and 65 ± 5% relative humidity.

4.2. Insects

A culture of the pea aphid, *A. pisum*, obtained from Biobest NV (Westerlo, Belgium) was maintained on broad bean (*Vicia faba*) plants. Tobacco aphids (*M. nicotianae*) were reared continuously on tobacco (*N. tabacum*) (Sadeghi et al., 2007). The two insect species were maintained in the Laboratory of Agrozoology at Ghent University, Belgium, under standardized conditions of 25 ± 2 °C, a 16 h photoperiod and 65 ± 5% relative humidity.

4.3. Bioassay with pea aphid in a feeding apparatus with artificial assay supplemented with purified SNA-I

SNA-I was purified from elderberry (*S. nigra*) bark to homogeneity using affinity chromatography on fetuin-Sepharose 4B and gel filtration, essentially as reported previously (Van Damme et al., 1996), and is essentially pure as judged by SDS–PAGE.

As food for the aphids, a standard diet previously developed for *A. pisum* (Febvaye et al., 1988) was used as the basal diet to which the purified SNA-I were added. Then the prepared liquid artificial diet was filter-sterilized through a 0.2 μm filter (Millipore Corp., Bedford, MA). Aliquots of 1.5 ml can be stored in the freezer at −20 °C for a period up to 6 months. After defrosting at room temperature, the artificial diet is ready to be used.

The feeding apparatus was prepared using plexiglass cylinders (3 cm high and 3 cm diameter). The food sachet was made under sterile conditions and consists of two layers of parafilm membrane on top of the container. About 200 μl of the artificial diet was sandwiched between the two layers.

To challenge aphids to SNA-I, a stock solution of the purified SNA-I was prepared in phosphate buffered saline (PBS), and then diluted in the artificial diet to prepare different RIP concentrations of 1, 5, 10, 25, 50, 100, 200, 400, 800, 1000 and 1200 μg/ml. In the treatments, 200 μl of each concentration was used to make a food sachet, whereas in the controls the diet was supplemented with PBS buffer. At day 0, 15 neonate nymphs (aged 0-24 h), obtained from a synchronized population reared on *V. faba* plants, were transferred onto the artificial diet. During the experiment the sachets were replaced every 2 days. For each concentration, four replicates were carried out and followed for 18 days. Aphids were checked daily for mortality to determine nymphal and adult survival and prereproductive period that is the period of time from birth until the onset of reproduction. Offspring produced in each replicate was recorded and removed daily. Recorded values were divided by the number of living adults in the corresponding replicate to evaluate total fecundity, and daily fecundity of each adult. The experiment was performed two times independently from each other.

4.4. Preparation of crude extracts and protein determinations with transgenic lines

Tissue samples from transformed and untransformed tobacco plants were lyophilized and homogenized in 20 mM 1,3-propane diamine (1.4 ml buffer per gram of fresh weight leaf material) with mortar and pestle. The homogenates were transferred to Eppendorf tubes and centrifuged (13,000 *g* for 5 min) and the supernatant stored in −20 °C until used. The protein concentration was determined using BIO-RAD protein assay kit (BIO-RAD, Hercules, CA).

4.5. Bioassay with tobacco aphids (M. nicotianae)

The effects of feeding *M. nicotianae* on the six transgenic lines and wild type tobacco plants were tested using bioassays in growth chamber conditions, essentially as described in Shahidi-Noghabi et al. (in press). In brief the test was conducted on detached leaves in Petri dishes (90 mm diameter). In the lids one hole was drilled for ventilation and covered by a net cloth. One randomly selected apterous female from the stock culture was transferred to an excised tobacco leaf (from 3 month-old plants) placed upside down on wet cotton wool. Thirty young aphids or neonates (aged 0–24 h) were selected and placed individually per Petri dish. This day is considered as day 0. The cotton in the Petri dishes was wetted daily and every 2 days the aphids were transferred to a new tobacco leaf. Nymphal development and survival were scored daily. The presence of exuvia was used to determine molting. The test was followed for 35 days, by which time no more nymphs were produced and daily reproduction of all individual adult aphids was recorded. At each daily count, newly deposited nymphs were removed with minimal disturbance to the adult. The experiment was performed two times independently from each other.

4.6. Life table analysis

Life table parameters were analyzed as described in Shahidi-Noghabi et al. (in press). Transgenic plants and the non-transformed wild type tobacco were used for insect feeding. The intrinsic rate of increase (r_m) is estimated by using iterative bisection method from $\sum_{x=0}^{\infty} e^{-r(x+1)} l_x m_x = 1$ with age indexed from 0 (Birch, 1948), where L_x is age-specific survival rate (x = age in days), M_x is age-specific fecundity (offspring/female/day). R_0 is the net reproductive rate, λ is finite rate of increase, T is the mean generation time. Prereproductive time (d) is the mean number of days from aphid birth to reproduction. The doubling time DT is the time required by the aphid population to double its size. Mean r_m values presented in Table 2 are the group values; standard errors were calculated using a jackknife estimator by successive suppression of one individual from group data (Miller, 1974).

4.7. Statistical analysis

Significantly different means were separated by using analysis of variance (ANOVA) followed by a *post-hoc* Tukey test, or a non-parametric Mann–Whitney *U*-test in SPSS v15 (SPSS, Chicago, IL). Survival analyses were performed using the standard comparison test of Wilcoxon in SPSS v15. Differences between treatments were considered significant at $p < 0.05$. The lethal concentration 50% (LC_{50}) was determined using the nonlinear regression analysis in Prism v4 (GraphPad, San Diego, CA).

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