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Ribosome-inactivating proteins from apple have strong aphicidal activity in artificial diet and *in planta*

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ABSTRACT

The apple (*Malus domestica*) genome contains gene sequences encoding type-1 and type-2 ribosome-inactivating proteins (RIPs). Both types of proteins contain a RIP domain with N-glycosidase activity, but the type-2 RIPs possess an additional domain with lectin activity. Here we investigated the activity of RIPs from apple against two sucking-piercing aphids that are important in agriculture, in particular pea aphids (*Acyrthosiphon pisum*) and green peach aphids (*Myzus persicae*).

When the recombinant RIPs were dosed in an artificial liquid diet against pea aphids a strong aphicidal activity was observed. Based on LC_{50}s, the type-2 RIP (33 mg/L) was about 10 times more active than the type-1 RIP (341 mg/L), and the LC_{50} of the recombinant protein corresponding to the type-2 RIP lectin domain alone was 106 mg/L. In *in planta* experiments with transgenic tobacco plants expressing the type-1 RIP or the type-2 RIP and infected with green peach aphids, mortalities of the nymphal stages amounted to approximately 50% for both types of transgenic lines when compared to wild type plants. In addition, significant sublethal effects were observed in the surviving aphids with a reduction in fecundity, intrinsic rate of increase, net reproductive rate and doubling time of the insect population. The insecticidal activity of the type-1 RIP and type-2 RIP from apple is discussed in relation to the domain structure of the RIPs and potential use in plant protection.

1. Introduction

Currently insect resistance is a problem for most of the synthetic chemical insecticides used in crop protection. Engineering of plants using genes encoding compounds with insecticidal activity has been reported as a valuable alternative to the use of chemical insecticides (Ranjekar et al., 2003; Gatehouse, 2008). Crops expressing the insecticidal protein from *Bacillus thuringiensis* (*Bt*) (Berliner) are commercially available for agricultural pest management since 1996 (James, 2013). However, two major problems occur with the *Bt* toxin-based technology. First, *Bt* toxin does not show protection towards sucking insects, and second, many insects developed a resistance to *Bt* toxin (Janmaat and Myers, 2003; Price and Gatehouse, 2008; Tabashnik et al., 2012, 2013; Bravo et al., 2015). Because of these problems there is a growing interest to look for alternative strategies based on the use of plant defense proteins.

The family of ribosome-inactivating proteins, abbreviated as RIPs, groups all enzymes (EC3.2.2.22) with a unique N-glycosidase activity that enables these proteins to depurinate a specific adenine residue from the highly conserved sarcin-ricin loop of the large ribosomal RNA, resulting in the catalytic inactivation of the ribosomes and a rapid arrest of cellular protein synthesis (Peumans et al., 2001; Stirpe and Battelli, 2006). Based on their structural organization the plant RIPs can be divided into two main groups (Peumans et al., 2001; Van Damme et al., 2001). Type-1 RIPs consist exclusively of a single N-glycosidase domain of approximately 30 kDa. In contrast, type-2 RIPs are composed of an N-terminal domain with enzymatic activity similar to type-1 RIPs and a C-terminal domain with lectin activity.

Though RIPs are widespread in the plant kingdom only little is known with respect to their importance for plant growth and development (Girbés et al., 2004). RIPs can vary from being non-toxic to extremely toxic proteins (Nielsen and Boston, 2001; Barbieri et al., 2004; Stirpe and Battelli, 2006). For instance, ricin, a RIP produced in castor beans (*Ricinus communis* L.), has high

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toxicity to mammalian cells and animals as well to insect pests (Olsnes, 1978; Gatehouse et al., 1990), while some type-2 RIPs from *Sambucus* species were reported as non-toxic RIPs for cultured animal cells and mice (Tejero et al., 2015).

In the last two decades several reports have put forward the idea that RIPs can enhance plant resistance to different pathogens and pests, including viruses, fungi as well as insects (Stirpe, 2013). Evidence was obtained from experiments with artificial diets supplemented with RIPs as well as from transgenic lines overexpressing a RIP gene. For instance, feeding of pea aphids (*Acyrthosiphon pisum* Harris) on artificial diet supplemented with SNA-I, a type-2 RIP from elderberry (*Sambucus nigra* L.) reduced survival and fecundity of this important aphid species (Shahidi-Noghabi et al., 2008). The same authors also showed that feeding of tobacco aphids (*Myzus nicotianae* Blackman) on leaves from tobacco plants expressing SNA-I delayed the development and reduced adult survival and fecundity. In addition, feeding of the soybean caterpillar (*Anticarsia gemmatalis* Hübner) and fall armyworm (*Spodoptera frugiperda* J.E. Smith) on a diet containing different type-1 RIPs (saporin, PAP-S, lychnin, gelonin and momordin) affected the survival and developmental rate of these important lepidopteran pests (Bertholdo-Vargas et al., 2009).

Recently RIPs have been reported in apple (*Malus domestica* Borkh) (Shang et al., 2013). Apple represents a nice model system in that both a type-1 and a type-2 RIP gene are present in the apple genome. In this study, we investigated the insecticidal activity of the type-1 and type-2 RIPs from apple against two aphid species that are important pests in agriculture and both have many populations with high levels of insecticide resistance against the commercial aphicides available. In a first set of experiments the purified recombinant proteins for type-1 RIP and type-2 RIP from apple were tested against pea aphids (*A. pisum*) using an artificial diet. In the second part of this study, tobacco plants expressing the apple RIPs were used to compare the insecticidal activity of type-1 and type-2 RIPs against green peach aphids (*Myzus persicae* Sulzer). The results are discussed in relation to the impact of the lectin domain for insecticidal activity of the RIP and the potential use of apple RIP sequences in the protection of crops against pest insects.

2. Materials and methods

2.1. Recombinant proteins

Recombinant type-1 RIP was purified from *Pichia pastoris* (Phaff) cells overexpressing the type-1 RIP sequence using hydrophobic interaction chromatography on Phenyl Sepharose (GE Healthcare, Uppsala, Sweden) and ion exchange chromatography on S Fast Flow (GE Healthcare). The type-2 RIP and its lectin domain were purified from transgenic tobacco Bright Yellow-2 cells constitutively expressing the recombinant the type-2 RIP or the type-2 RIP lectin domain using hydrophobic interaction chromatography combined with affinity chromatography on Fetuin-Sepharose (Shang, 2015). The purity of the recombinant proteins was judged by SDS-PAGE.

2.2. Insects

Continuous colonies of pea aphids (*A. pisum*) and green peach aphids (*M. persicae*) were maintained on young broad bean plants (*Vicia faba* L.) and tobacco plants (*Nicotiana tabacum* L. cv Samsun NN), respectively, at the Laboratory of Agrozoology, Ghent University, Belgium, under standardized conditions of 25 ± 2 °C, 16 h light:8 h dark photoperiod and 65 ± 5% relative humidity (Sadeghi et al., 2009; Shahidi-Noghabi et al., 2009).

2.3. Plants

Transgenic tobacco (*N. tabacum* cv Samsun NN) plants expressing the type-1 RIP or type-2 RIP from apple have been constructed (Shang, 2015). To obtain a constitutive expression of the RIP, the binary constructs are driven by the 35 S cauliflower mosaic virus promoter. Wild type tobacco plants and different transgenic tobacco lines (T1 generation, type-1 RIP lines 3, 6, 7 and 23, and type-2 RIP lines 11, 22, 28 and 44) were used to perform the insect bioassays. Tobacco seeds were sterilized with 80% ethanol (v/v) and sown in petri dishes containing MS medium (Murashige and Skoog, 1962). After two weeks, the germinated seedlings were transferred to soil. Leaves of plants 6–10 weeks old were used for the insect bioassays.

2.4. Insect bioassays with pea aphids (A. pisum) on artificial diet using recombinant proteins

To synchronize the age of the nymphs, mature aphids were put on a bean plant, and after 24 h, all neonate nymphs were used in the bioassay. The basal food used for the aphids was developed from a standard diet for *A. pisum* as described in Sadeghi et al. (2009). In brief, a feeding sachet was constructed by putting 150 μl artificial diet in between two layers of parafilm under sterile conditions. To determine the effects of the proteins (Type-1 RIP, Type-2 RIP or Type-2 RIP lectin domain) on the neonate nymphs, several concentrations of the recombinant proteins (Type-1 RIP: 80–960 mg/L; Type-2 RIP: 17–204 mg/L; Type-2 RIP lectin domain: 30–360 mg/L) were tested. Since a solution of 20 mM un-buffered diaminopropane was used to dissolve the recombinant proteins, an artificial diet supplemented with equal volumes of this solution (max. 7%) was used as a control. A total number of ten neonate nymphs was transferred onto the artificial diet containing the recombinant proteins or the control diet. The mortality was determined at 24 h-intervals, and the dead insects were removed daily for three consecutive days. For each protein concentration, three replicates were carried out. The 50% lethal concentration (LC_{50}) together with the 95% confidence limits and the R^2 of the sigmoid curve fitting were determined using the non-linear regression analysis in Prism version 5 (GraphPad, La Jolla, CA).

2.5. Bioassay with green peach aphids (M. persicae) on tobacco plants overexpressing RIPs

The effects of feeding *M. persicae* on transgenic lines and wild type tobacco plants were tested using bioassays under standard conditions in an incubator (Versatile Environmental Test Chamber, Panasonic Healthcare, Osaka, Japan) at 25 °C, 60% relative humidity and a 16:8 (light:dark) photoperiod. The insect bioassay was conducted with detached leaves in insect breeding dishes (100 × 40 mm) (SPL Lifesciences, Gyeonggi-do, S-Korea), as reported before (Shahidi-Noghabi et al., 2009). In brief, in the lid of the petri dish there is a hole for ventilation (40 mm) covered by a net cloth. Leaves of similar age were used for the bioassay (the fourth or fifth leaves located at the top of the plants). To synchronize the age of the nymphs, adults were transferred to fresh tobacco plants to give birth to neonate nymphs. Next day, a first instar nymph (aged 0–24 h) was placed individually in an insect breeding dish that contains a piece of tobacco plant leaf (about 4 cm^2) placed upside down on wet cotton wool. This day is considered as day 0. Fifteen nymphs were used per line or wild type. The cotton in the Petri dishes was wetted daily and every 2 days the piece of the tobacco leaf was replaced by fresh one. Nymphal development and survival were scored daily. The presence of exuvia (aphid molted cuticle) was used as evidence of molting to determine the aphid