Identification of S-RNase and peroxidase in petunia nectar

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Previous SDS PAGE gel analysis of the floral nectars from petunia and tobacco plants revealed significant differences in the protein patterns. Petunia floral nectar was shown to contain a number of RNase activities by in gel RNase activity assay. To identify these proteins in more detail, the bands with RNase activity were excised from gel and subjected to trypsin digestion followed by LC–MS/MS analysis. This analysis revealed that S-RNases accumulate in nectar from Petunia hybrida, where they should carry out a biological function different from self-pollen rejection. In addition, other proteins were identified by the LC–MS/MS analysis. These proteins include a peroxidase, an endochitinase, and a putative fructokinase. Each of these proteins contained a secretory signal sequence that marked them as potential nectar proteins. We developed RT–PCR assays for each of these five proteins and demonstrated that each of these proteins was expressed in the petunia floral nectary. A discussion of the role of these proteins in antimicrobial activity in nectar is presented.

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Introduction

Floral nectar is a secretion composed mainly of sugars and amino acids that is used by plants as a reward to attract pollinators. Many other substances also accumulate in nectar, including alkaloids, coumarins, saponins, and non-protein amino acids that may render nectar toxic or repellent to some animals. These compounds have been shown to deter nectar thieves but not legitimate pollinators (reviewed by Nicolson and Thornburg, 2007).

Proteins are also present in nectar. However, in spite of extensive analyses of nectar chemical composition, little is known about the floral nectar proteome (reviewed by Nicolson and Thornburg, 2007; Nepi et al., 2009). An exception is the nectar from ornamental tobacco. Ornamental tobacco nectar contains five main nectarins, NEC I–V, that have been well characterized (reviewed by Nicolson and Thornburg, 2007). NEC I, II and III are part of a biochemical pathway termed the nectar redox cycle that protects nectar and the gynoecium from attack by microorganisms (Carter and Thornburg, 2004a). This pathway produces high levels of hydrogen peroxide via two independent mechanisms (Carter and Thornburg, 2000, 2004b). The accumulation of hydrogen peroxide is the primary antimicrobial defense of tobacco nectar, since nectar treated with catalase becomes a good substrate for microbial growth (Carter et al., 2007).

In contrast to tobacco, and in spite of a close phylogenetic relationship, the protein profiles of petunia and tobacco nectars are very different, and petunia nectar does not rely on H₂O₂ for antimicrobial protection (Hillwig et al., 2010). Biochemical analyses of petunia nectar detected a large number of ribonuclease (RNase) activities that are not present in tobacco (Hillwig et al., 2010) and could have a defensive role. These analyses led to the identification of novel RNases belonging to the RNase T2 family that are expressed in the nectaries of petunia plants (Hillwig et al., 2010). In plants, this family of RNases is divided into two classes, S-RNases and S-like RNases. S-RNases are secreted into style mucilage, where they abort the growth of pollen bearing the same S-allele as the pistil (Clarke and Newbigin, 1993), while S-like RNases are proposed to function in inorganic phosphate recycling and in defense against pathogens (Bariola and Green, 1997; Deshpande and Shankar, 2002). Two nectary-expressed RNases from petunia, RNase Phy3 and RNase Phy4, were identified (Hillwig et al., 2010). Although their expression in nectaries and the presence of secretion signal peptides suggest that these proteins may be secreted into nectar, these RNases cannot account for all the RNase activities that were detected in petunia nectar.

To identify other RNases present in petunia nectar, we took advantage of a well established in gel RNase activity assay that allows for separation of enzymes while retaining activity. After separation, these proteins can be recovered from the acrylamide gel and subjected to protein sequencing. With this approach we
identified S-RNases and defense proteins present in petunia nectar.

Materials and methods

Plant material, protein and RNA preparations

Petunia hybrida plants were obtained from a local market. Plants were grown to floral maturity in a greenhouse with supplemental light (16 h day/8 h night). Nectar was collected as described by Carter et al. (1999), 6 h after watering to ensure adequate nectar production. Nectar was also obtained from the ornamental tobacco hybrid LxS8 (Nicotiana langsdorfii × Nicotiana sanderae var LxS8). Nectar from different plants was combined, and the samples were maintained at –80 °C until used. To analyze proteins, nectar was diluted 1:1 with water. RNA from different plant tissues was prepared as described by Hillwig et al. (2010).

Protein separation, sequencing and bioinformatics

Nectar proteins were separated by one dimensional 10% SDS-PAGE. Fifty microlitres of nectar was analyzed (nectar protein concentration of 0.1–0.2 mg mL−1). After separation, gels were stained with Coomassie Blue using GelCode Blue Stain Reagent (Pierce/Thermo Scientific) according to manufacturer recommendations, or silver-stained according to published procedures (Blum et al., 1987). The same amount of nectar was also analyzed by an in gel RNase activity assay as described previously (Hillwig et al., 2010). After separation, the main band from the Coomassie-stained SDS-PAGE and the seven most prominent RNase bands from the in gel assay were manually excised. These protein bands were submitted to the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center, St. Louis, MO, for further analysis. There, in gel trypsin digestion and nano-LC–MS/MS were performed as described by Alvarez et al. (2009).

For peptide identification, all MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot). Mascot was set up to search the NCBI nr database, and queried with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 0.80 Da. Oxidation of methionine was specified in Mascot as a variable modification. Scaffold (version Scaffold_2.05.02, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if the corresponding peptides were established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003).

Three independent experiments were carried out. The proteins identified were similar in each experiment.

Expression analysis

Proteins identified by proteomics analysis were used as query to search the NCBI nucleotide nr database and the est, others database with TBLASTN, limiting the search to sequences for the genus Petunia. When available, a full-length cDNA was used to design primers for PCR. If this option was not available, the longest possible cDNA was assembled using EST sequences. A table with the primers used is included as Supplementary Table 1. These primers were used to perform RT–PCR using RNA prepared from different petunia tissues, as described by Hillwig et al. (2010). PCR products were submitted for sequencing to the Iowa State University DNA Facility to confirm their identity. The experiments shown are representative of two independent experiments with triplicate assays.

Peroxidase and chitinase assays

Peroxidase and chitinase assays were carried out according to the methods described by González-Teuber et al. (2009). Standards used to calculate enzyme activities were Horseradish peroxidase (Sigma, catalog number P-6782) and Streptomyces griseus chitinase (Sigma, catalog number C-6137).

Sugar analysis

Determination of the main carbohydrate composition of nectar was carried out by thin-layer chromatography as described by White and Robyt (1988). Two microlitres of a 1:10 dilution of nectar in water was analyzed. The result is representative of two independent experiments.

Results

In order to characterize RNase activities present in petunia nectar, we decided to separate proteins in a semi-denaturing acrylamide gel containing high molecular weight RNA, and those with RNase activity were subjected to protein sequencing. After separation, gels were incubated to allow for renaturation of RNases resulting in regeneration of RNase activity. Finally, RNA was stained. Portions of the gel that contained RNases showed reduced staining that corresponded to RNase activity (Fig. 1). At least seven well defined RNase activities were detected in the gel, and some of their properties have been described earlier (Hillwig et al., 2010). Semi-denaturing conditions (no reducing agents or heat treatments) were used because heat denaturation in the presence of strong reducing agents results in complete loss of RNase activities (Supplementary Fig. 1). After separation, bands were excised (indicated by boxes in Fig. 1) and analyzed as described in “Materials and Methods” section.

In addition, since one major band can be identified in denaturing SDS-PAGE analyses of petunia nectar (Fig. 1, see also additional Coomassie and silver-stained gels in Supplementary Fig. 2), we decided to excise this band from a SDS-PAGE gel and it was also submitted to nanoLC–MS/MS analysis. In addition to protein identification, a comparison of the number of identified tandem mass spectra assigned to each protein after normalizing by molecular weight provided a rough estimate of the relative abundance of the different proteins in cases of co-migration (Zybailov et al., 2005).

Several proteins were identified by this approach (Supplementary Table 2). We found two RNases among the proteins separated in the zymogram. These RNases belong to the S-RNase class of RNases. Both S1-RNase and Sx-RNase are alleles from the S-locus in petunia. We also identified a peroxidase and an endochitinase, proteins that have a role in defense against microorganisms. Finally, we found several proteins normally associated with housekeeping roles, including ribosomal proteins and enzymes involved in glycolysis. The most abundant protein based on spectra counting was the secretory peroxidase (not shown), followed by a putative fructokinase. Intriguingly, the gene encoding for this fructokinase is genetically linked to the S-locus that encodes for S-RNases in petunia (Wang et al., 2003).

Several of the proteins identified by peptide sequencing have housekeeping roles and are normally localized in either plastids or the cytoplasm. Finding them in secreted nectar was unexpected. Nectar proteins, secreted from the nectaries, are expected to be targeted to the secretory pathway via secretory signal peptides. Therefore, to validate the identification of proteins as bona fide nectarins we looked for predicted signal peptides for the peptides identified in Supplementary Table 2. Only the first five proteins were predicted to have a secretion signal peptide, the others were
Fig. 1. RNase and protein profile of petunia nectar. Aliquots (50 μL) of raw nectar from Petunia hybrida were analyzed in an in gel RNase activity assay (left panel), or by SDS-PAGE and stained with Coomassie Blue (right panel). N, petunia nectar; M, molecular weight markers. Bands that were excised for proteomics analysis are indicated by boxes and numbered. Gels are representative of at least 3 independent experiments.

Fig. 2. Expression of petunia nectarins in different tissues. Flowers were harvested at stage 12 (before dehiscence), and dissected to obtain sepals (Se), petals (P), anthers (A), stigmas (St), styles (Sty), gynoecia (ovaries plus nectaries, G), and nectaries (N). At the same time, leaves (L), stems (S) and roots (R) were collected. Expression of the five RNase genes was analyzed by RT-PCR. Amplification of 18S was used as control for loading. Gels are representative of at least 3 independent experiments.

Detection of enzymatic activities in nectar also confirmed the presence of these proteins in nectar. We have previously characterized RNase activities in nectar (Hillwig et al., 2010), and these activities are shown in Fig. 1. Peroxidase seems to be the most abundant protein in petunia nectar according to our sequencing analysis. In contrast, and in spite of extensive characterization, peroxidase protein or activity has not been detected in tobacco nectar. Thus, we compared peroxidase activity in petunia and tobacco nectars. We detected a strong peroxidase activity in petunia nectar (Fig. 3A, note the logarithmic scale), at least three orders of magnitude stronger than the weak activity found in tobacco. Weak chitinase activity was also detected in both petunia and tobacco nectars (Fig. 3B).

Fig. 3. Analysis of peroxidase and chitinase activity in petunia and tobacco nectars. (A) Aliquots of raw nectar from petunia or ornamental tobacco were tested for peroxidase activity in vitro. Tobacco nectar was used directly; petunia nectar was diluted 1:100 before analysis. Note the logarithmic scale. (B) The same samples (without dilutions) were analyzed for chitinase activity in vitro.
It is not clear whether the other proteins identified by sequencing are also nectarins or contaminants. Presence of several glycolytic enzymes in nectar would indicate that nectar sugars may be processed once they are secreted. However, a sugar analysis of petunia nectar (Supplementary Fig. 3) indicated that sugars in petunia nectar were not degraded, and the ratio sucrose:glucose:fructose was similar to that described before (Stuurman et al., 2004) and also relatively similar to that from tobacco (Supplementary Fig. 3 and Ren et al., 2007). However, the ratio glucose:fructose seems to be different from 1:1, with less fructose than glucose accumulating in petunia nectar.

Discussion

We identified two RNase proteins from nectar samples that had not previously been associated with this secretion. S-RNases are members of the RNase T2 family, and are involved in the process of self-incompatibility in at least three plant families. These RNases inhibit pollen tube growth when pollen and pistil share the same allele in the S-locus (Clarke and Newbiggin, 1993). It has been hypothesized that this cytotoxic function of S-RNases evolved from an RNase that had a defensive function in flowers (Hiscock et al., 1996; Nasrallah, 2005). This defense role would be similar to that proposed for S-like RNases in other plants (Machnosh et al., 2010). Although expression of petunia S-RNases has been detected in non-stylar tissue (Clarke and Sims, 1994), a function for this RNase outside of its self-incompatibility role has not been identified, but based on these results, should be considered. Here we show expression in nectaries, and accumulation of the S-RNAses in nectar, suggesting that S-RNases still perform a defensive function against microorganisms, supporting the hypothesis that their self-incompatibility function evolved from a defense role.

In addition, we detected the accumulation of a secreted peroxidase and a secreted endochitinase. We also found a strong peroxidase activity in petunia nectar. These proteins are also associated with antimicrobial plant defense mechanisms. In this regard, petunia nectar seems to be similar to extraloral nectar from several species of Acacia (González-Teuber et al., 2009) as well as the reproductive pollination drops of Douglas Fir (Pouls et al., 2005; O’Leary et al., 2007). Accumulation of pathogenesis-related proteins, including peroxidase and chitinases, is thought to protect these plant secretions from microbial infestation (Nepi et al., 2009; Gonzalez-Teuber et al., 2010).

It is unclear why we failed to detect other RNase proteins in bands with stronger RNase activity than the S-RNases. It is possible that these proteins are present in low amounts, although they apparently have high activity. In this case, additional separation protocols should be implemented to obtain larger amounts of the RNase without other proteins that might interfere with LC–MS/MS identification. It is also possible that we could not identify some peptides due to incomplete information on the petunia genome since it has not been fully sequenced. Intriguingly, the most abundant protein in some of the bands showing RNase activity is a peroxidase. Previously, Xing et al. (2005) attempted to identify an RNase activity using proteomics. While their approach failed to do so, some of the proteins identified in the bands with RNase activity were also peroxidases. More than a decade ago, an animal polysomal RNase was shown to be a novel member of the peroxidase family (Chernokalskaya et al., 1998); thus, it could be possible that the RNase, or other of the proteins identified in our study may have a dual function including an RNase activity. Although this is a highly speculative hypothesis, it opens a new area of investigation.

The role of fructokinase in nectar is not clear. This protein also seems to have a secretory signal peptide, shows good expression in nectaries, and was one of the most abundant proteins in our sample. While the main role of fructokinase is to mediate fructose metabolism intracellularly, it has been proposed that this enzyme could have a signaling role similar to hexokinase (Pego and Smeekens, 2000). Remarkably, petunia nectar seems to have lower proportion of fructose than glucose. It is known that invertases have a role in regulating the ratio sucrose:monosaccharides in nectar (Heil et al., 2005; Nepi et al., 2009), and it has been proposed that the same kind of enzymes are responsible for the maintenance of osmotic gradients necessary for nectar secretion (Ruhland et al., 2010). However, the mechanisms that control the ratio fructose:glucose, which should be 1:1 if only invertases are involved in regulating sugar composition of nectar, are less understood. Recently, an elegant model suggesting that the monosaccharides are independently secreted by nectaries was used to explain fructose:glucose ratios in Antigozanthos flavidus (Wenzler et al., 2008). Whether fructokinase is secreted and somehow regulates fructose levels in nectar need to be investigated further.

Alternatively, the unbalanced glucose:fructose ratio could be the result of the activity of microorganisms present in nectar. Nectar sugar composition is affected by nectar colonization by yeast (Herrera et al., 2008), a microorganism commonly found in nectar of many plant species (Herrera et al., 2009). Microorganism contamination could also explain some of the non-secretory proteins found in our analysis. However, yeast colonization is associated with insect-mediated pollination, and our plants were maintained in greenhouses and were not pollinated. Thus, microorganism contamination due to contact with insects should be minimal.

In conclusion, we used protein sequencing to identify petunia nectar proteins. We were able to show that S-RNases do indeed accumulate in nectar, where they probably function in defense against microorganisms in conjunction with other defense proteins, like peroxidase and endochitinase, also identified by our approach. This analysis shows that in spite of their close phylogenetic relationship, petunia and tobacco nectars are indeed very different.

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Appendix A. Supplementary data


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