

RESEARCH PAPER

Petunia nectar proteins have ribonuclease activity

Melissa S. Hillwig, Xiaoteng Liu, Guangyu Liu, Robert W. Thornburg* and Gustavo C. MacIntosh*

Department of Biochemistry, Biophysics, and Molecular Biology, 2214 Molecular Biology Building, Iowa State University, Ames, Iowa 50011 USA

* To whom correspondence should be addressed: E-mail: Robert Thornburg: thorn@iastate.edu; Gustavo MacIntosh: gustavo@iastate.edu

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Abstract

Plants requiring an insect pollinator often produce nectar as a reward for the pollinator's visitations. This rich secretion needs mechanisms to inhibit microbial growth. In *Nicotiana* spp. nectar, anti-microbial activity is due to the production of hydrogen peroxide. In a close relative, *Petunia hybrida*, limited production of hydrogen peroxide was found; yet petunia nectar still has anti-bacterial properties, suggesting that a different mechanism may exist for this inhibition. The nectar proteins of petunia plants were compared with those of ornamental tobacco and significant differences were found in protein profiles and function between these two closely related species. Among those proteins, RNase activities unique to petunia nectar were identified. The genes corresponding to four RNase T2 proteins from *Petunia hybrida* that show unique expression patterns in different plant tissues were cloned. Two of these enzymes, RNase Phy3 and RNase Phy4 are unique among the T2 family and contain characteristics similar to both S- and S-like RNases. Analysis of amino acid patterns suggest that these proteins are an intermediate between S- and S-like RNases, and support the hypothesis that S-RNases evolved from defence RNases expressed in floral parts. This is the first report of RNase activities in nectar.

Key words: Nectar, nectarin, nectary, petunia, ribonuclease, RNase T2.

Introduction

In many angiosperms, male and female sexual organs are physically located in different places on the flower or on different flowers entirely; and many of these plants rely on animal pollinators to transfer pollen between flowers. Often these visiting pollinators are insects, however, birds, mammals, and even reptiles are known to function in pollen transfer among flowers. The visiting pollinators do not, however, do this for free. Instead, plants offer the visiting pollinators an incentive in return for pollen transfer. This reward consists of nectar, a rich concoction of sugars, amino acids, vitamins, lipids, and proteins (Nicolson and Thornburg, 2007), that is freely offered to attract the pollinators to the flower where pollen transfer takes place. The composition of floral nectar suggests that it may be a good growth medium.

Floral nectar is produced from a novel floral organ termed the nectary that is generally located inside the flower, usually at its base. When pollinators scavenge inside the flower for nectar they inadvertently pick up pollen

grains and transfer them when they change flowers. However, these visiting pollinators are also a hazard to the plant. By freely ranging between the reproductive tracts of many flowers, pollinators also transfer micro-organisms between flowers.

However, infections of the flower are rare in plants. Initial observations identified an array of five nectarins (nectar proteins) that were secreted into the nectar of ornamental tobacco plants (Carter *et al.*, 1999) and led to the hypothesis that a major function of the nectary is to protect the gynoecium from micro-organisms vectored to the flower by visiting pollinators (Thornburg *et al.*, 2003). Isolation and characterization of these proteins (Carter and Thornburg, 2000, 2004*b*, *c*; Naqvi *et al.*, 2005), helped define a novel biochemical pathway, the nectar redox cycle (Carter and Thornburg, 2004*a*), that exists in soluble floral nectar of ornamental tobacco. This pathway produces high levels of hydrogen peroxide (up to 4 mM; (Carter and Thornburg, 2000)) via two independent mechanisms. The

nectar redox cycle begins with the developmental expression of an NADPH oxidase in the floral nectary (Carter *et al.*, 2007). NADPH oxidase produces superoxide at the nectary membrane surface. Subsequently, the superoxide dismutase Nectarin I (NEC1), the major nectar protein, directly converts superoxide into hydrogen peroxide (Carter and Thornburg, 2000). This accumulation of hydrogen peroxide is the main antimicrobial defence of tobacco nectar, since nectar treated with catalase becomes a good substrate for microbial growth (Carter *et al.*, 2007).

The production of a superoxide dismutase protein as a mechanism of floral defence against microbes is well established in tobacco plants (Carter *et al.*, 2007). The nectar proteins have been characterized from only a few species of plants. In leek (*Allium porrum*), two nectar proteins have been characterized. The first is a mannose-binding lectin and the second is alliinase (Peumans *et al.*, 1997). Proteins in these families have anti-herbivore and antimicrobial properties, suggesting a defensive role for the leek nectar proteins as well. Characterization of *Jacaranda mimosifolia* nectar identified a nectar lipase that also appears to participate in defence (Kram *et al.*, 2008).

Recently, nectarins have also been identified in extrafloral nectar. In *Acacia* spp. an invertase was identified in soluble extrafloral nectar that modified the hexose/sucrose ratio to benefit associated ant species (Heil *et al.*, 2005); and later, classical defence proteins such as the pathogenesis-related PR proteins were identified in the extrafloral nectar of these plants (Gonzalez-Teuber *et al.*, 2009). Further, the reproductive secretions of gymnosperms have also been examined and found to contain both carbohydrate-modifying enzymes and defence proteins (Poulis *et al.*, 2005; O'Leary *et al.*, 2007; Wagner *et al.*, 2007). These findings suggest that the defence of plant secretions is an important and ancient feature of plant biology.

While preliminary studies predicted that the presence of NEC1 in nectar may be widespread among the angiosperms (Carter and Thornburg, 2000), this has never been directly tested and the occurrence of many different defence proteins in other species suggest that perhaps there are many ways to protect nectar from microbial invasion. This can only be addressed by examining nectar defence mechanisms from other closely related species. Therefore, the nectarins of a species (hybrid petunia) that is related to ornamental tobacco have been examined. These studies, outlined below, indicate that the nectar of petunia has a novel defence that is not related to that found in ornamental tobacco, but may be mediated by ribonucleases; furthermore, nectar defences based upon H₂O₂ may not be as highly conserved as previously thought.

Ribonucleases (RNases) are proteins that have the ability to degrade RNA. There are many different classes of RNases, all members of families with specific substrate preferences and enzymatic properties (D'Alessio and Riordan, 1997; Mishra, 2002). Ribonucleases belonging to the RNase T2 family are among those proteins enriched in flower tissues and may also have a defensive

role. These proteins are normally found in the secretory pathway and many accumulate in the extracellular space (Irie, 1999; Deshpande and Shankar, 2002). The S-like RNases, a subclass of RNase T2 enzymes found in all plant species (MacIntosh *et al.*, 2010), are commonly expressed in flowers. The three characterized S-like RNases from *Arabidopsis*, *RNSI-3*, are expressed at a higher level in flowers than in any other tissue (Taylor *et al.*, 1993; Bariola *et al.*, 1994, 1999), with *RNSI* being detected only in flowers in the absence of stress (Bariola *et al.*, 1994). Many other S-like RNases have been isolated from flowers, or cloned from pistil libraries, or their expression has been detected mainly in flowers in a diversity of species, including tobacco *RNase NE* (Dodds *et al.*, 1996), *Antirrhinum AhSL28*, an S-like RNase from Japanese pear styles (Norioka *et al.*, 2007) among others.

S-like RNases are proposed to function in two main physiological processes: nutrition, through the recycling of inorganic phosphate during periods of phosphate starvation or during senescence and other developmental stages involving cell-death; and defence against pathogens (Bariola and Green, 1997; Deshpande and Shankar, 2002). S-RNases are the other class of RNase T2 enzymes found in flowers. S-RNases participate in gametophytic self-incompatibility in at least three plant families (Hua *et al.*, 2008). S-RNases are secreted into style mucilage, where they abort the growth of pollen bearing the same S-allele (Clarke and Newbigin, 1993). This cytotoxic activity and their expression in flowers lead to the hypothesis that gametophytic self-incompatibility may have evolved through the recruitment of an ancient flower ribonuclease involved in defence mechanisms against pathogens for use in defence against 'invasion' by self pollen tubes (Hiscock *et al.*, 1996; Nasrallah, 2005).

Tobacco nectar has been well characterized. In addition to the identification of the defence mechanism and main protein complement of tobacco nectar, the biochemical changes and key regulators of gene expression controlling nectary development and nectar secretions have been characterized (Horner *et al.*, 2007; Ren *et al.*, 2007a, b; Liu *et al.*, 2009). However, knowledge on the conservation of these mechanisms in nectar from other related species is lacking. In a first attempt to extend the characterization of nectar to other species, an analysis is presented here of nectar proteins from *Petunia hybrida*, which, like tobacco, belongs to the Solanaceae family. Petunia nectar has potent antimicrobial activity, but surprisingly does not produce large amounts of hydrogen peroxide, although petunia and tobacco are closely related species. Instead, petunia nectar contains many ribonuclease activities not found in tobacco. Novel RNase T2 enzymes expressed in nectaries with characteristics intermediate between S- and S-like RNases were identified. These proteins could represent an intermediate step in the evolution of S-RNases, and support the hypothesis that S-RNases were recruited for self-incompatibility participation from an ancestral defence-related role in flowers.

Materials and methods

Plant material

Petunia hybrida plants were obtained from a local market. *Nicotiana tabacum* cv. Xanthi was obtained from Dr CA Ryan, Washington State University. The ornamental tobacco hybrid L×S8 (*Nicotiana langsdorffii*×*Nicotiana sanderae* var. L×S8) was described previously (Kornaga *et al.*, 1997; Carter *et al.*, 1999). Plants were grown to floral maturity in a greenhouse with supplemental light (16/8 h day/night). Nectar was collected as described in Carter *et al.* (1999) approximately 6 h after watering to ensure adequate nectar production. For RNA and protein extraction, tissues from different floral parts were harvested at the appropriate floral stage following the classification of Koltunov *et al.* (1990).

FOX assay for hydrogen peroxide

Hydrogen peroxide was assayed in nectar essentially as described by Bleau *et al.* (1998). Briefly, 1 ml of fresh FOX reagent (25 mM sulphuric acid, 100 µM xylenol orange, 100 µM D-sorbitol, and 250 µM ferrous ammonium sulphate) was added to 200 µl of diluted nectar. After incubating for 20 min at room temperature, the levels of hydrogen peroxide were quantitated spectroscopically at 560 nm and calculated using a hydrogen peroxide standard curve (up to 300 µM).

Bactericidal assay

Raw nectar was diluted 1:1 with 10 mM phosphate buffer (pH 7.0) to improve pipetting precision. A pH of 7.0 was used because this is the normal pH of petunia nectar [determined with pH indicator strips (Merck)]. A fraction of the nectar was treated with catalase (Sigma) as described in Carter *et al.* (2007) for 20 min. Then, 90 µl aliquots of filter-sterilized nectar were used to test bacterial growth in a 96-well microplate. *Pseudomonas fluorescens* (strain A506) was grown in LB overnight at 28 °C in the presence of 50 mg l⁻¹ rifampicin. The bacterial culture was then diluted to an OD₆₀₀=0.5 using Luria Broth (LB). Ten µl of culture were added to each microplate well containing nectar from ornamental tobacco L×S8, *Petunia hybrida*, or phosphate buffer; with or without catalase treatment. Triplicate plates were incubated in a plate reader with agitation for 18 h at 28 °C and the OD₆₀₀ was measured every 30 min. Growth was normalized (to *t*=0 for each well). Each treatment was assayed a minimum of three times.

In vitro gel assay

Raw nectar was collected from *Petunia hybrida*, *Nicotiana tabacum* cv. Xanthi, and ornamental tobacco plants L×S8, and stored at -80 °C until use. Fifty µl of nectar were analysed on RNase and DNase activity gels as described by Yen and Green (1991). Due to the presence of a compound that interfered with our standard method for protein quantification, the amount of protein loaded was estimated based on comparisons of stained proteins with molecular markers of known concentration. This estimate indicated that 5–10 µg of nectar proteins were loaded in each lane. For tissue-specific protein analysis a minimum of six flowers (stage 12) were dissected to obtain sepals, petals, stamens, stigmas, styles, and ovaries (including nectaries). Tissue was ground using a mortar and pestle with liquid N₂, and extracted as described by MacIntosh *et al.* (1996), except that the extraction buffer did not include polyvinyl polypyrrolidone and 2-mercaptoethanol. Protein concentration was determined using the Bio-Rad Protein Assay Kit, and 100 µg of total protein were analysed in RNase or DNase activity gels. For anther/stamen analysis, at least six flowers at each stage (2, 6, 9, 11, 12a, 12b) were collected and stamens were harvested for protein isolation as stated above. Each activity gel is a representative of two independent protein isolations, and at least three replicates.

Protein integrity was determined by SDS-PAGE analysis. After electrophoresis, gels were stained with Coomassie Blue using GelCode Blue Stain Reagent (Pierce/Thermo Scientific) according to the manufacturer's recommendations.

Cloning of RNases

Nectaries were isolated from *Petunia hybrida* flowers as described for ornamental tobacco (Carter *et al.*, 1999). RNA was extracted from ovaries and nectaries using the Qiagen RNeasy Plant Mini Kit, and cDNA was synthesized using the i-Script Select Kit (Bio-Rad). To amplify cDNAs corresponding to RNase T2 homologues, primers were designed corresponding to conserved nucleotide regions by comparing sequences from *Arabidopsis RNS1* (Taylor and Green, 1991), tobacco *RNase NE* (Dodds *et al.*, 1996), and petunia *RNase X2* (Lee *et al.*, 1992). Primers were also designed based on petunia ESTs with homology to RNase T2 sequences. The primers used are presented in Supplementary Table S1 at JXB online. PCR products were cloned into pGEM T-EASY or pGEM T vector (Promega) for sequencing purposes. RNase Phy3 and RNase Phy4 were subjected to rapid amplification of cDNA ends (RACE)-PCR using the GeneRacer Kit (Invitrogen). DNAs were sequenced at the Iowa State University DNA Facility. The petunia RNase clone sequences were deposited in GenBank as accessions GQ465917 to GQ465920.

RT-PCR

Sepals, petals, stamens, stigma, styles, ovaries (with nectaries), nectaries alone, leaves, roots, and stems from *Petunia hybrida* were collected, and RNA was extracted as described above. Genomic DNA was removed using a DNA-free kit (Applied Biosystems), and cDNA was synthesized using the i-Script Select Kit (Bio-Rad). PCR was performed using GoTAQ 2X Master Mix (Promega) and 35 cycles of PCR products were run on 1% TBE gels and stained with ethidium bromide. Amplification of 18S RNA was used as control for loading.

Phylogenetic analysis

Protein sequences were aligned using the CLC bio software package, followed by manual adjustments. Only the region between the first conserved region after the signal peptide and the last conserved C residue was used in phylogenetic analyses. PAUP 4.0 software (Swofford, 2002) was used for Neighbor-Joining (1000 bootstrap replications) and parsimony analyses, using default parameters.

Results

Antimicrobial activity of Petunia hybrida nectar is not based on H₂O₂ production

Ornamental tobacco nectaries are bright orange (Fig. 1a) due to the accumulation of β-carotene (Horner *et al.*, 2007). The increase in nectary carotenoids is concomitant with the accumulation of H₂O₂ in the nectar (Carter and Thornburg, 2004a; Horner *et al.*, 2007); and it is proposed that the production of β-carotene and ascorbic acid provides the counter-balancing antioxidants needed to protect nectary cells, and probably the rest of the gynoeceum, from the highly oxidative environment caused by H₂O₂ (Horner *et al.*, 2007).

A direct comparison of the nectaries of *Petunia hybrida* with those of the ornamental tobacco hybrid L×S8 (*Nicotiana langsdorffii*×*Nicotiana sanderae* var. L×S8)

showed that, in contrast to the ornamental tobacco, the mature nectaries of *Petunia hybrida* do not turn bright orange, but rather remain a dull yellow. This observation suggested that the biochemical processes occurring in tobacco and petunia nectaries could be different, and that

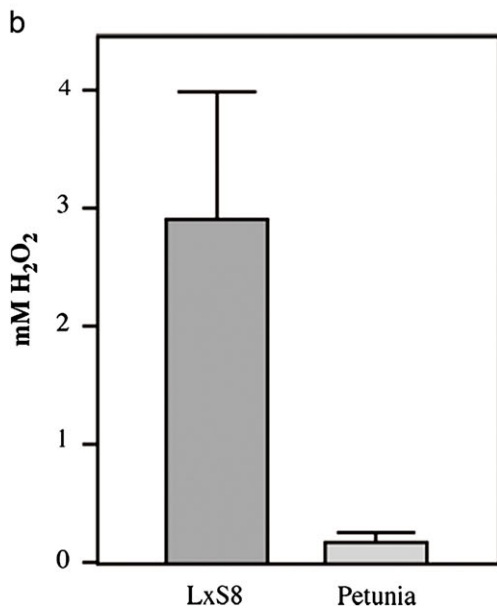
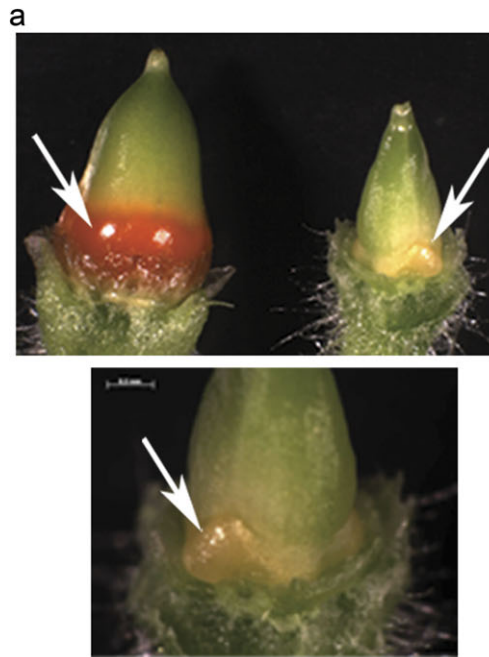


Fig. 1. Differences in nectary appearance and nectar composition between petunia and tobacco. (a) Appearance of petunia (right in upper panel, and lower panel) and the LxS8 tobacco hybrid (left, upper panel) nectaries (arrows) from flowers at stage 12 (Koltunow *et al.*, 1990). Observe the differences in size and colour; small, light yellow nectaries in petunia, large, bright orange nectaries in tobacco. (b) Accumulation of hydrogen peroxide in petunia and tobacco nectar. Nectar collected from at least 20 different flowers was pooled and analysed for the presence of H₂O₂ using a colorimetric assay.

petunia may use different mechanisms of defence against micro-organisms. To test this idea, nectar was collected from both species and their H₂O₂ content was measured (Fig. 1b). Tobacco nectar accumulates up to 4 mM H₂O₂, as previously reported by Carter and Thornburg (2004b). On the other hand, H₂O₂ accumulation in petunia nectar is more than 10-fold lower than in tobacco.

The nectar of ornamental tobacco effectively inhibits the growth of micro-organisms (Carter *et al.*, 2007). This inhibition depends on the production of H₂O₂, and it is lost if nectar is treated with catalase. It was found that *Petunia hybrida* nectar also possesses antimicrobial activity. *Petunia* nectar can inhibit the growth of *Pseudomonas fluorescens*, *Salmonella typhimurium*, and *Erwinia amylovora* (data not shown). *Petunia hybrida* nectar contains low levels of H₂O₂; however, it could still be enough to provide antimicrobial protection. To test whether H₂O₂ was involved in this antimicrobial effect, the inhibitory effect of petunia and ornamental tobacco (LxS8), with or without prior treatment with catalase, was compared. The bacteria *Pseudomonas fluorescens* strain A506 was used in this assay because it had previously been shown to be inhibited by LxS8 tobacco nectar (Carter *et al.*, 2007). Figure 2 shows that both tobacco and petunia nectar inhibit the growth of *P. fluorescens*. However, this inhibition is significantly reduced after catalase treatment of ornamental tobacco nectar. On the other hand, catalase treatment had no effect on the petunia nectar, which was still capable of inhibiting bacterial growth. This result suggests that a H₂O₂-independent antimicrobial mechanism exists in petunia nectar.

To determine whether the potency of the antimicrobial activity of petunia nectar is comparable with that of tobacco, dilutions were made of both nectars and their ability to support *P. fluorescens* growth was determined. While half-strength petunia nectar is more effective than tobacco nectar at inhibiting bacterial growth, a one-sixth

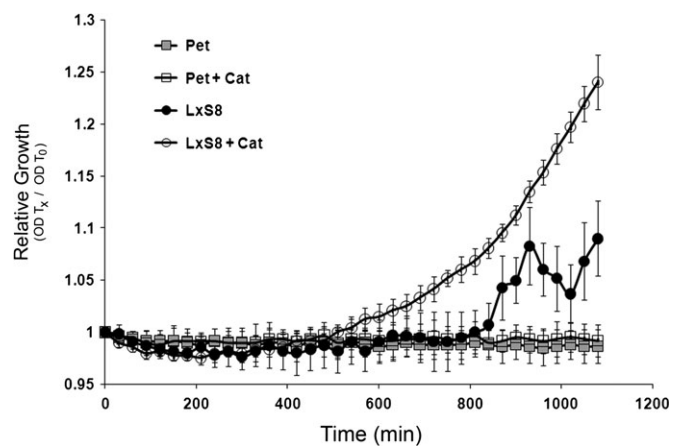


Fig. 2. Effect of tobacco (circles) and petunia (boxes) nectar on the growth of bacteria. Growth of *Pseudomonas fluorescens* (strain A506) in raw nectar (filled symbols) or nectar that was preincubated with catalase (empty symbols) was followed by changes in OD. Each point represents the mean \pm SD ($n=3$). Data are representative of two independent experiments.

dilution of petunia and tobacco nectars support the same level of growth (see Supplementary Fig. S1 at *JXB* online).

Petunia nectar is rich in ribonuclease activities

Because other defensive mechanisms are suggested in petunia nectar and RNases are commonly found in flowers, it was decided to look for ribonuclease activities in nectar. To determine if RNases are present in the nectar of the tobacco and petunia plants an *in gel* activity assay was used (Yen and Green, 1991). Nectars from *Petunia hybrida* and two tobacco species (*Nicotiana tabacum* cv. Xanthi, and the ornamental tobacco hybrid L×S8) were collected and analysed on SDS-PAGE gels in which RNA was included. After electrophoresis the gels were incubated at three different pHs to improve the chance of detecting any RNases present. These assays detected RNase activities in all nectar samples (Fig. 3a); and, in general, RNases present in the nectar of all species had higher activity at an acidic pH. However, petunia showed a more complex RNase profile. At least 8–10 bands were detected in the petunia nectar, ranging from ~20–40 kDa. By contrast, only two bands were detected in L×S8 (~20 kDa and 25 kDa) and an additional 1–2 weak bands in *N. tabacum* cv. Xanthi in the same size range.

The estimated protein concentration of tobacco and petunia nectar is in the same range (0.1–0.2 mg ml⁻¹); however, it could be possible that some of the observed differences are the result of different amounts of proteins in the samples analysed. Thus, an RNase activity assay using similar amounts of protein from each sample was performed (see Supplementary Fig. S2 at *JXB* online). Again, petunia nectar showed a large number of RNase activities that were not present in tobacco.

The different nectar samples were also tested for deoxyribonuclease (DNase) activities by the *in gel* activity assay (Fig. 3b). Three DNase activities were identified in petunia nectar. Two bands (approximately 30 kDa and 38 kDa) seem to coincide with RNase activities and show similar pH preference in DNA and RNA gels, suggesting that these two enzymes are bifunctional nucleases. Another activity of ~25 kDa seems to be a basic DNase only observed in petunia nectar. By contrast, no DNase activity was detected in the ornamental tobacco nectar and a single activity at ~37 kDa was found in the *N. tabacum* nectar.

The differences in RNase and DNase activities between petunia and tobacco nectars are not due to protein degradation in the samples, since the protein profiles determined by Coomassie Blue and silver staining did not show signs of proteolysis (Fig. 3c). The nectarin profile of ornamental tobacco shows the major NEC1 protein at ~29 kDa (Carter and Thornburg, 2000) and the NEC4/NEC5 doublet at ~65 kDa (Carter and Thornburg, 2004c; Naqvi *et al.*, 2005). NEC3 (40 kDa) and its breakdown product, NEC2 (35 kDa) are often difficult to observe (Carter and Thornburg, 2004b). The *N. tabacum* nectar shows the NEC1 and the NEC4/NEC5 doublet and a number of minor bands. By contrast, the nectarin profile of petunia is clearly different from that found in either of the two tobacco

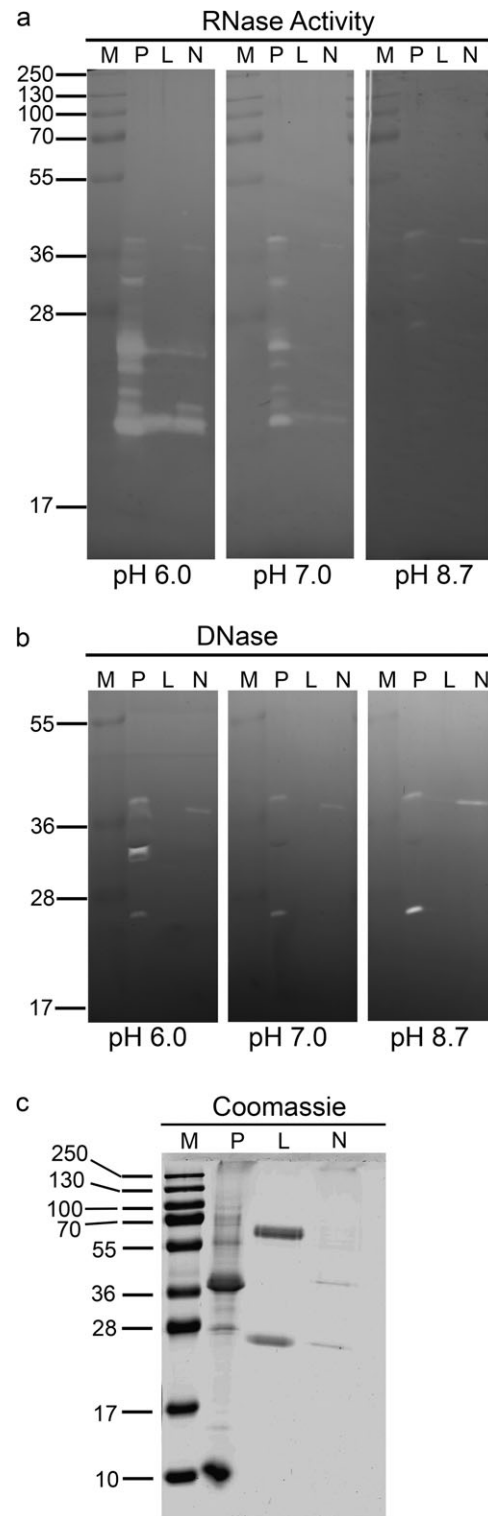


Fig. 3. Nuclease activities are present in nectar. (a) Aliquots (50 μ l) of raw nectar from *Petunia hybrida* and two different tobaccos (*Nicotiana tabacum* cv. Xanthi and the hybrid *Nicotiana langsdorfii* × *Nicotiana sanderae* var. L×S8) were analysed in an *in gel* RNase activity assay at three different pHs. P, petunia; L, L×S8; N, Xanthi. Size (kDa) of molecular weight markers (M) is indicated. (b) Same samples as in (a), but analysed in an *in gel* DNase activity assay. (c) The same samples as in (a), analysed by SDS-PAGE, and stained with Coomassie Blue. Gels are representative of at least three independent experiments.

species analysed. The two major proteins migrate at ~10 kDa and 38 kDa. At least four minor bands at approximately 28 kDa, 32 kDa, 56 kDa, and 70 kDa are also present in petunia nectar.

To determine if the RNases present in the nectar of petunia plants were expressed solely in the nectar or were also found in other parts of the flower as well, protein extracts from different flowers parts were assayed. Petunia and ornamental tobacco flowers were dissected into six primary organs; sepal, petal, stamens, stigma, style, and ovary (including nectaries). Protein extracts were prepared from these samples and run on RNase (Fig. 4a) and DNase (Fig. 4b) activity gels at pH 6.0. As shown in Fig. 4a, it is evident that each floral organ in the two species shows a different RNase profile. Petunia has a very complex pattern of activities in the 20–27 kDa range, and few activities larger than 27 kDa. On the other hand, ornamental tobacco flowers have a series of activities in the 27–38 kDa range not observed in petunia, but lack many of the activities in the smaller range (Fig. 4a). Many of the largest sized activities seem to coincide with DNase activities (Fig. 4b). While only one DNase activity was identified in petunia samples, up to six different bands can be seen in the various tobacco floral organs. Similarities in pattern of expression and relative intensity suggest that most of the activities detected in the 27–38 kDa range correspond to bifunctional nucleases, with the exception of an activity of ~33 kDa expressed only in petunia stigmas and styles that clearly has only RNase activity.

Several of the smaller RNases that are enriched in petunia seem to accumulate preferentially in the reproductive organs rather than in sepals and petals. Activities of ~18, 18.5, and 20 kDa are present only in stamens (anthers+filaments), stigmas, styles, and ovaries; and an activity of ~22.5 kDa is present in all samples, but is highly enriched in stamens, stigmas, and styles.

The stamens from both petunia and ornamental tobacco flowers contained the largest number of RNase activities as well as the most abundant DNase activity. Increased expression of RNases has been observed during senescence (Taylor *et al.*, 1993; Liang *et al.*, 2002; Lers *et al.*, 2006). Thus, to determine if this increase in activities was due to senescence (dehiscence) of the anthers, proteins from anthers at various stages of flower development were prepared and analysed on RNase activity gels (Fig. 5). From our analysis it is clear that most RNases present in anthers are expressed during all stages of development and are not induced during senescence, i.e. no differences were observed between anthers from stage 12a (before dehiscence) and 12b (after dehiscence). However, the 18 kDa and 18.5 kDa doublet of activities increases during anther development, while some activities in the 30–40 kDa range are only observed in the early stages.

Novel RNase T2 genes are expressed in petunia nectaries

Since RNase T2 enzymes are commonly found in flowers, a search was made for this type of transcript in petunia

nectaries. RNA from isolated nectaries and ovaries was prepared and RT-PCR was used to amplify transcripts belonging to this family. BLASTP searches of the non-redundant protein database of NCBI identified many petunia S-RNases, but no petunia S-like RNases. It is hypothesized that any RNase T2 enzyme in nectar would belong to the S-like RNase class, since this class has been implicated in plant defence. Primers were designed based on

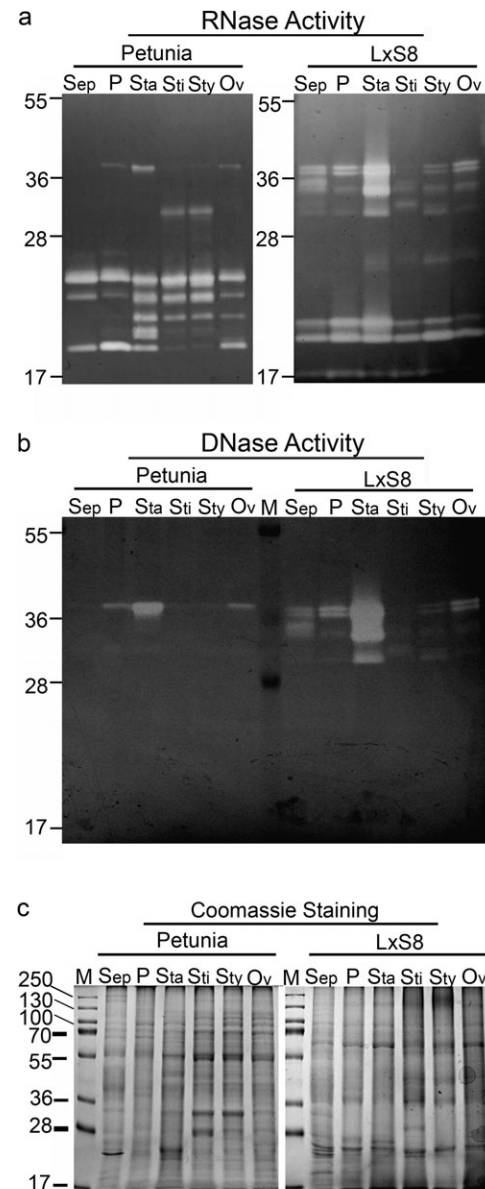


Fig. 4. Nuclease profiles of different floral parts of petunia and ornamental tobacco plants. Flowers were harvested at stage 12, and dissected to obtain sepals (Sep), petals (P), stamens (Sta), stigmas (Sti), styles (Sty), and ovaries (including nectaries, Ov). Total protein extracts (100 µg) from each floral part were analysed in an *in gel* RNase activity assay (a) or DNase activity assay (b) at pH 6.0. (c) Same samples as in (a) analysed by SDS-PAGE, and stained with Coomassie Blue. Position of molecular weight markers (kDa) is indicated. Gels are representative of at least three independent experiments.

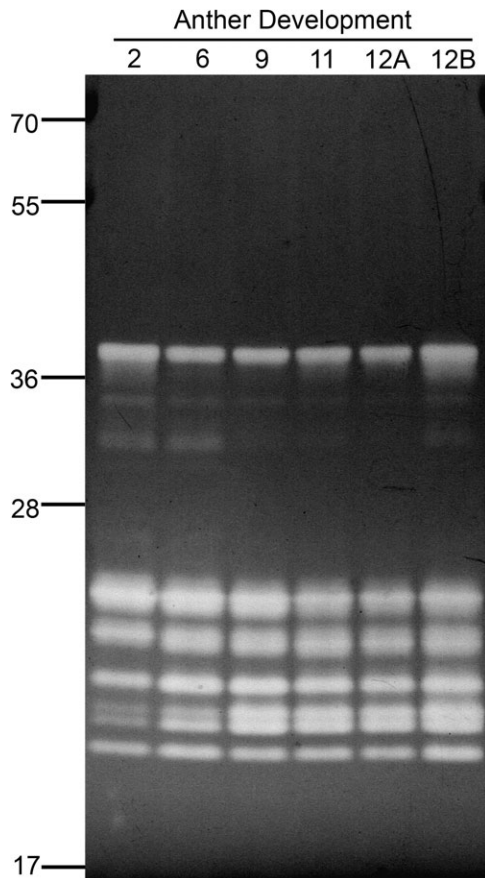


Fig. 5. RNase profile of petunia stamens during development. Stamens were collected from flowers at pre-dehiscence (2, 6, 9, 11, 12A) and post-dehiscence (12B) stages. Total protein extracts (100 µg) were analysed in an *in gel* RNase activity assay at pH 7.0. Position of molecular weight markers (kDa) is indicated. Gel is representative of at least three independent experiments.

conserved regions of S-like RNases, determined by sequence alignment of RNaseNE (GenBank accession number AAA21135), RNaseLX (GenBank accession number P80196), and RNS1 (GenBank accession number P42813). We also searched for petunia ESTs that could correspond to RNase T2 enzymes, and primers were designed to amplify these sequences. Primer sequences are presented in Supplementary Table S1 at *JXB* online.

Using different primer combinations it was possible to amplify four distinct sequences that contained the conserved active site (CAS) cassettes that define enzymes belonging to the RNase T2 family (Irie, 1999). These were named *RNase Phy1*, *RNase Phy3*, *RNase Phy4*, and *RNase Phy5*, and were deposited in the GenBank as accessions GQ465920, GQ465919, GQ465918, and GQ465917, respectively. BLASTP analysis (Fig. 6) of the predicted proteins encoded by these partial sequences indicated that RNase Phy1 has 96% similarity and 90% identity to RNase NE from tobacco. Likewise, RNase Phy5 showed high homology (95% similarity, 88% identity) to tomato RNase LX. However, BLAST analyses of RNase Phy3 and RNase Phy4 resulted in hits with low sequence homology, either at the nucleotide or the amino acid levels. The closest homologue to RNase Phy3

was also RNase NE, but with only 33% identity and 52% similarity, and large gaps. The closest homologue to RNase Phy4 was an S-RNase, S42-RNase from *Pyrus × bretschneideri*, and the homology was even lower than for RNase Phy3 (29% identity, 48% similarity). In both cases homology was higher around the two CAS that define this family of enzymes. Due to their unique sequences *RNase Phy3* and *RNase Phy4* were subsequently chosen for rapid amplification of cDNA ends (RACE) analysis to determine their complete transcript sequence.

RACE PCR analysis of *RNase Phy3* yielded a partial transcript. 5' RACE was unsuccessful in yielding a complete 5' end; however, sequencing analysis did reveal the first and second CAS sites. The partial *RNase Phy3* transcript is 639 nucleotides long. The predicted protein encoded by this gene has an estimated molecular weight of 23.8 kDa, and an isoelectric point of 9.25, and it is probably N-glycosylated. RACE PCR of *RNase Phy4* yielded a full-length transcript of 861 nucleotides. The encoded protein showed a putative signal peptide of 19 aa. The molecular weight of the mature protein is 25.79 kDa, with an isoelectric point of 8.98. RNase Phy4 may have up to three possible N-glycosylation sites. RNase Phy3 has a 38% identity and a 63% similarity with RNase Phy4. BLASTP analyses (not shown) indicated that these two proteins have similar homology to S-RNases and S-like RNases, and are not clear members of either class.

Tobacco nectarins are expressed exclusively in nectararies that are actively secreting nectar (NEC1, NEC4, and NEC5; (Carter and Thornburg, 2003, 2004c; Naqvi *et al.*, 2005)) or in nectararies and a few other floral tissues (NEC3; Carter and Thornburg, 2004b). The four petunia RNases were cloned from nectary and/or ovary cDNA. To analyse whether their expression was limited to these organs or found throughout the plant, RNA was extracted from different flower and vegetative tissues and tested for the presence of the corresponding transcripts using RT-PCR (Fig. 7). Each of the four RNases was expressed in ovaries and, in addition, *RNases Phy1*, 3, and 4 were also detected in nectararies. *RNase Phy1* was expressed ubiquitously throughout the plant, and although our analysis is only semi-quantitative, its expression does seem higher in floral organs than in vegetative tissues. *RNase Phy3* and *RNase Phy4* had similar expression profiles. Both were expressed exclusively in flowers, with strong expression in ovaries and nectararies. *RNase Phy4* was also highly expressed in petals and weakly detected in styles, while *RNase Phy3* was highly expressed in stigmas, but also was detected in styles and petals. *RNase Phy5* was mostly expressed in styles, although weak expression was also observed in petals, stamens (anthers), and ovaries. Thus, only *RNase Phy3* and *RNase Phy4* have patterns consistent with that of nectarins. These results suggest a role for these proteins in nectar.

RNase Phy3 and *RNase Phy4* have characteristics of S- and S-like RNases

Plant members of the RNase T2 family are classified in three groups based on their phylogenetic relationships, their

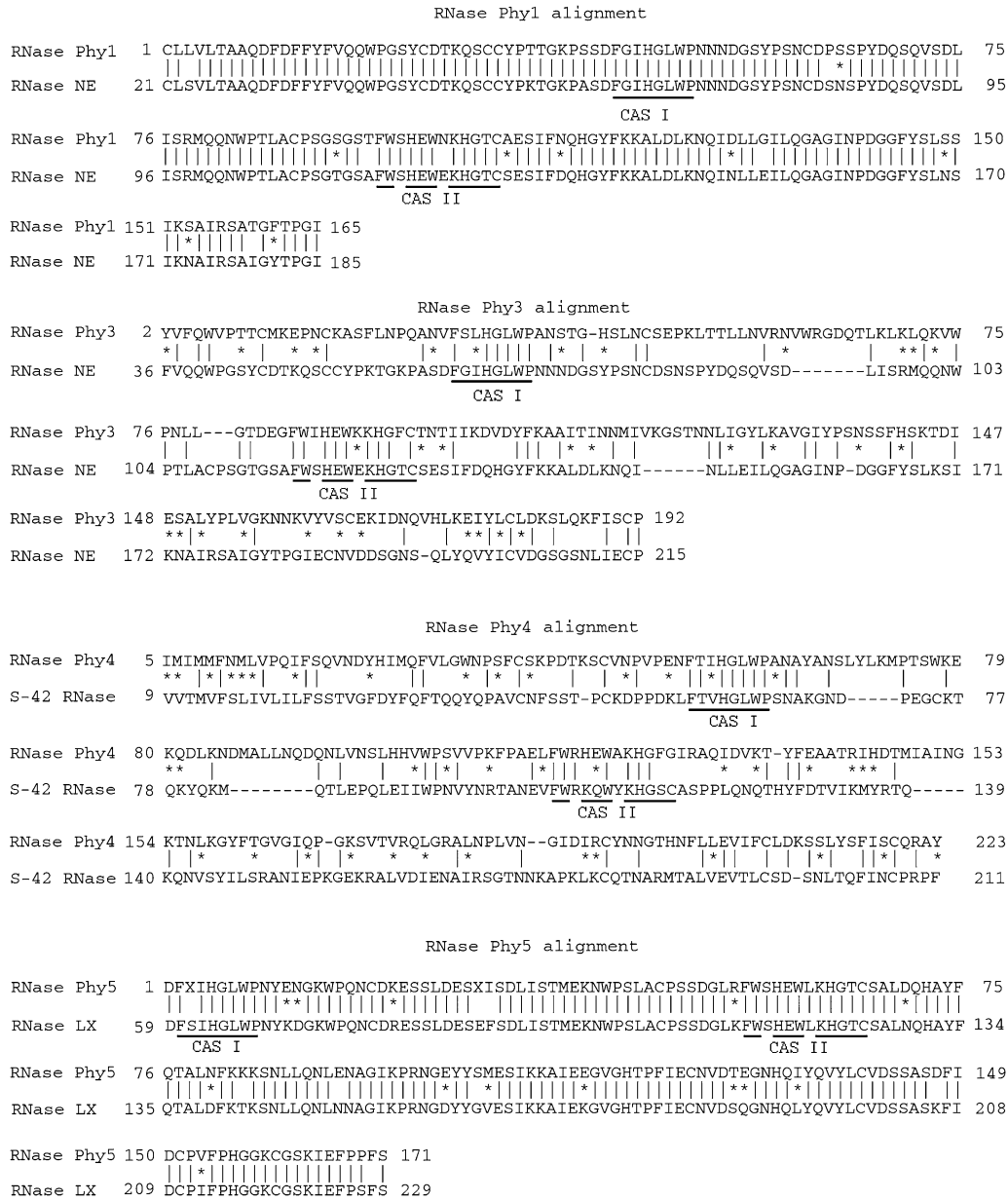


Fig. 6. Petunia RNases have homology to RNase T2 enzymes from other plants. BLAST analysis of predicted RNases encoded by petunia cDNAs amplified from ovaries and nectaries RNA. Alignment of each petunia RNase (RNase Phy1, RNase Phy3, RNase Phy4, and RNase Phy5) with the homologue with the highest BLAST score is shown.

protein properties and their genomic organization (Igc and Kohn, 2001; MacIntosh *et al.*, 2010). Classes I and II include the S-like RNases, which, in general, are acidic enzymes with either less than four introns (Class I) or more than four introns (Class II). Class III includes S-RNases, ‘relic’ S-RNases (Golz *et al.*, 1998), and other RNases that have been proposed as ancestors of S-RNases (Yamane *et al.*, 2003). Relic S-RNases are believed to have originated from the duplication of S-RNase genes but do not participate in self-incompatibility. Most S-RNases and relic S-RNases are basic proteins and have only one intron, with the exception that S-RNases of the genus *Prunus* have two introns (Yamane *et al.*, 2003). RNase Phy3 and RNase

Phy4 show low homology to both S-like and S-RNases; and they have characteristics from each of these classes. These two petunia RNases are basic proteins, as are most S-RNases; but their expression patterns do not resemble S-RNases, which are expressed mainly in the pistil. By contrast, RNase Phy3 and RNase Phy4 are also found in nectaries, ovaries, and petals.

Amino acid patterns have also been used to differentiate between S-like and S-RNases. Vieira *et al.* (2008) described four amino acid patterns that can be used to distinguish between these two classes of RNases. Two patterns were identified exclusively in S-RNases (patterns 1 and 2, shaded yellow in Fig. 8), and also two were used to define S-like

RNases (patterns 3 and 4, shaded pink in Fig. 8). In their analysis Vieira *et al.* identified pattern 1 in 467 of 468 S-RNases analysed, while pattern 2 was found in 689 of 691 possible S-RNase sequences. On the other hand, the amino acid pattern [HY]EW (pattern 3) was found in 54 of 69

S-like RNases and but only in 7 of 658 S-RNase sequences (each of these seven sequences belonged to the genus *Prunus*), and pattern 4 was found in 64 of 69 S-like RNases studied, and was not found in any of the 658 S-RNase sequences used in that study (Vieira *et al.*, 2008).

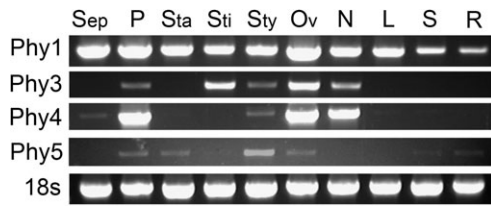


Fig. 7. Expression of petunia RNases in different flower parts. Flowers were harvested at stage 12, and dissected to obtain sepals (Sep), petals (P), stamens (Sta), stigmas (Sti), styles (Sty), ovaries (including nectaries, Ov), and nectaries (N). At the same time, leaves (L), stems (S), and roots (R) were collected. Expression of the four RNase genes was analysed by RT-PCR. Amplification of 18S was used as control for loading. Gels are representative of at least three independent experiments.

RNase Phy1 and RNase Phy5 contain the two S-like RNase patterns (Fig. 8). However, RNase Phy3 and RNase Phy4 do not match either class. RNase Phy3 contains patterns 2 and 3, corresponding to S- and S-like RNases, respectively (Fig. 8). RNase Phy4 contains only pattern 3, indicative of S-like RNases (Fig. 8), but does not have pattern 4. Thus, RNase Phy3 and RNase Phy4 show characteristics that are intermediate between S-RNases and S-like RNases, although RNase Phy4 seems to be closer to S-like RNases.

A phylogenetic analysis of plant RNase T2 proteins was performed to determine the relationship of RNase Phy3 and RNase Phy4 with other RNases in this family. A Neighbor-Joining tree is shown in Fig. 9. This tree included proteins belonging to the three classes, as previously analysed by MacIntosh *et al.* (2010), with the addition of petunia

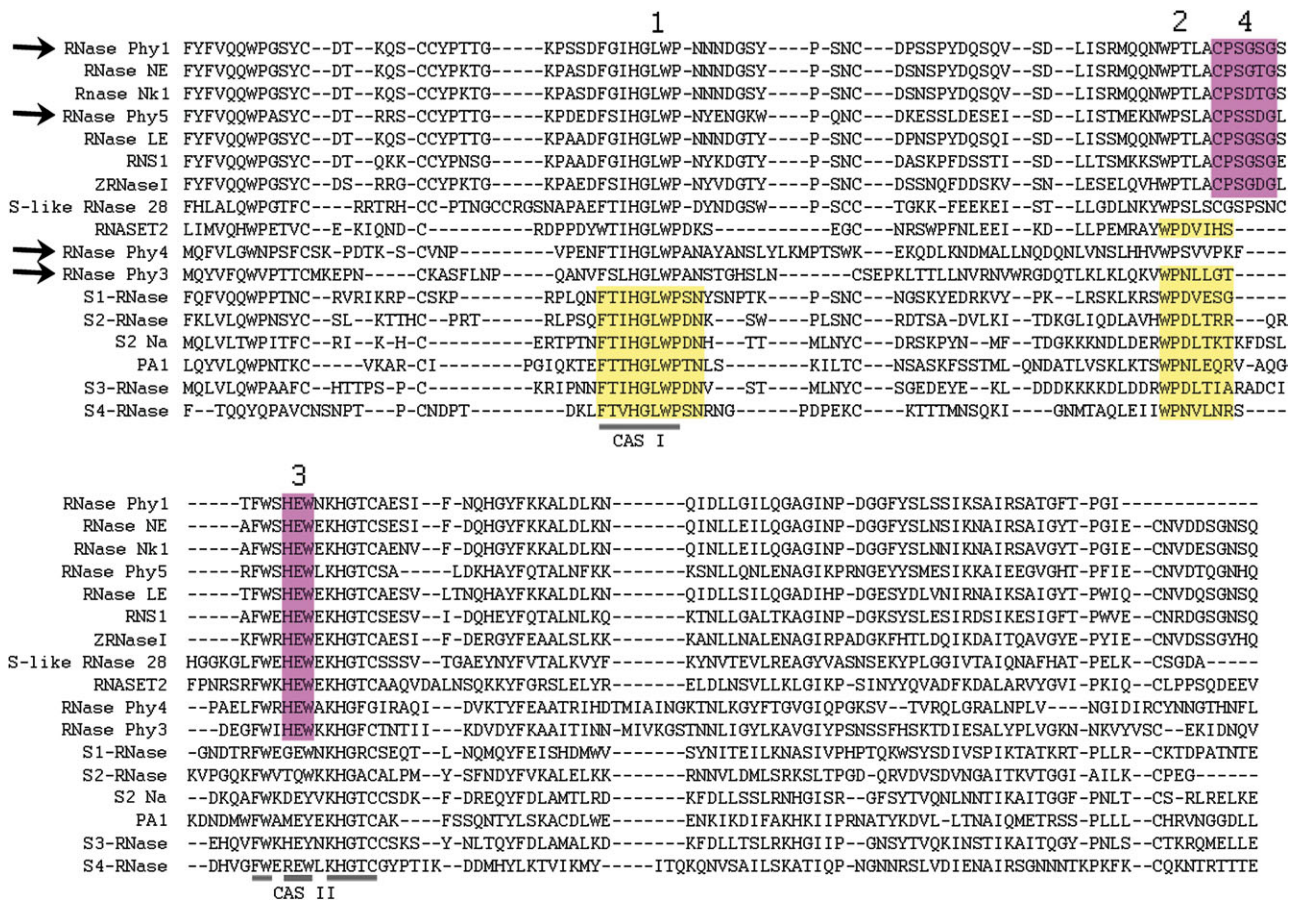


Fig. 8. Presence of S- and S-like RNase-specific patterns (according to (Vieira *et al.*, 2008)) in petunia RNases. Alignment of the petunia RNases and representative members of the S-RNase and the S-like RNase subfamilies. Patterns 1 and 2 that define S-RNases are highlighted in yellow; S-like RNase patterns are pink. The conserved active sites (CAS) I and II, typical of RNase T2 enzymes, are indicated. Petunia RNases are indicated with arrows. Accession number of other S-like RNase proteins in the alignment are AAA21135 (RNase NE), BAA95448 (RNase Nk1), X79337 (RNase LE), P42813 (RNS1), AAC49325 (ZRNaseI), CAC50874 (S-like RNase 28); S-RNases included are BAA83479 (S1-RNase), CAA65319 (S2-RNase), AAB40027 (S2 Na), BAD11006 (PA1), AAB07492 (S3-RNase), and BAA28354 (S4-RNase). We also included NP_003721 (RNASET2) from *Homo sapiens*.

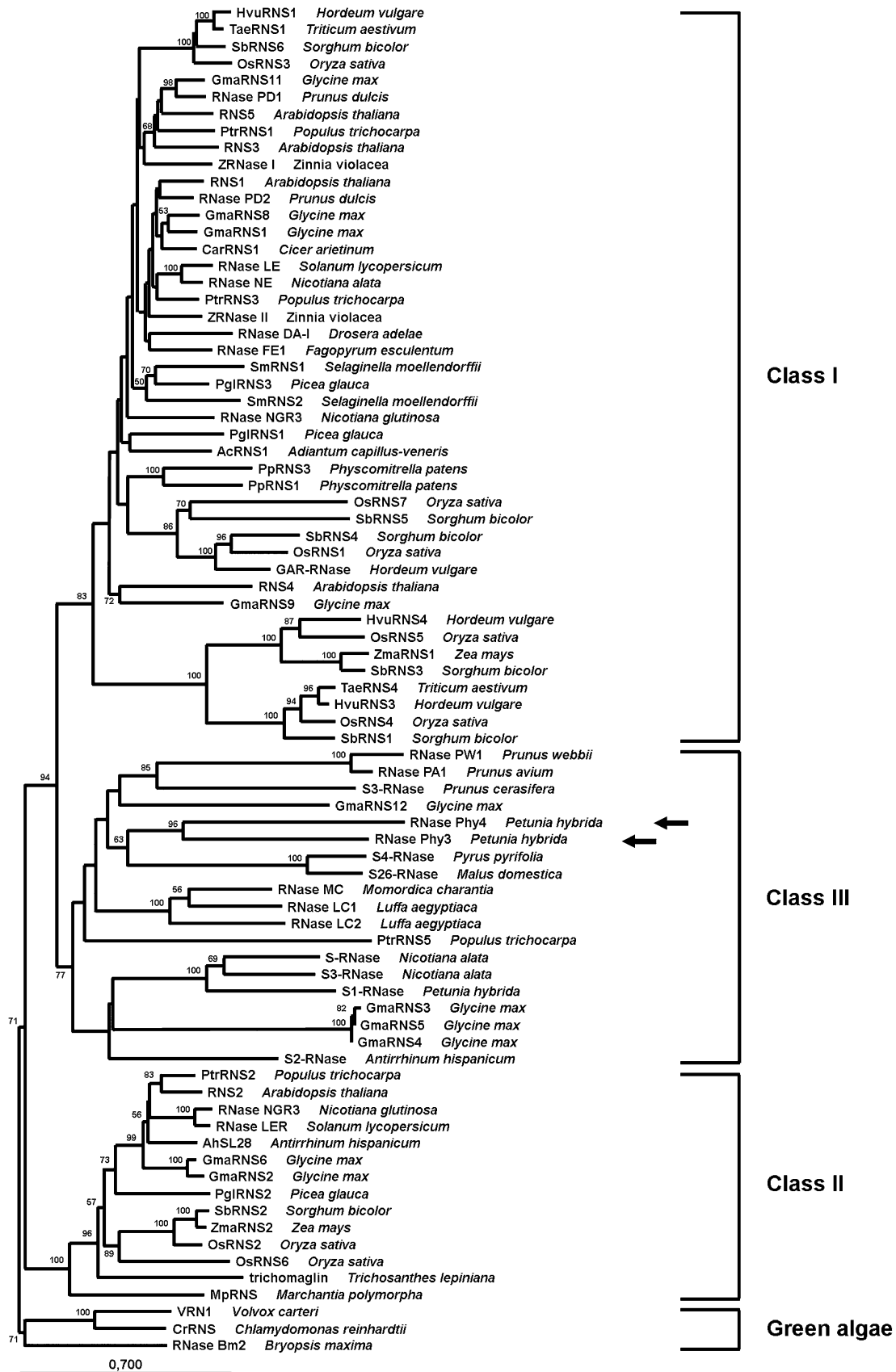


Fig. 9. Phylogenetic relationship of plant RNase T2 proteins. The Neighbor–Joining tree was estimated using only conserved regions of plant RNase T2 proteins. Bootstraps percentages greater than 50 are shown on interior branches. The tree was rooted using algae sequences. Classes I, II, and II clades are indicated, as well as algae proteins. Accession numbers of proteins included in the tree are those described in MacIntosh et al. (2010), with the addition of RNase Phy3 (arrow), RNase Phy4 (arrow), RNase PW1 (ABY86422), RNase PA1 (BAD11006), S3-RNase from *P. cerasifera* (CAN90133), S4-RNase (BAA28354), S26-RNase (AAB70515), S-RNase (BAA24017), S3-RNase from *N. alata* (AAB07492), S1-RNase (AAA60465), and S2-RNase (CAA65319).

RNases and canonical S-RNases. As expected, three clades are defined, each corresponding to one of the plant RNase T2 classes previously described. Although the bootstrap support for each clade is very strong, the internal architecture of the individual clades for Class I and Class III is less supported. RNase Phy3 and RNase Phy4 clearly belong to Class III, which includes canonical S-RNases and other RNases believed to have derived from ancestral RNases that gave origin to S-RNases, or from relic RNases that may have lost their self-incompatibility function (MacIntosh *et al.*, 2010). Surprisingly, these two petunia RNases seem to be closer to RNases found in the Rosaceae than to other Solanaceae proteins. Moreover, RNase Phy3 and RNase Phy4 are very different from the canonical S-RNases found in *Petunia hybrida* (Fig. 9; and data not shown).

Discussion

Although the importance of nectar in pollination is well-recognized, the proteins that are present in this plant secretion and, in particular, the proteins involved in antimicrobial activities are, in general, not well-studied. The best-studied example is the nectar from ornamental tobacco. Several nectarins, proteins present in nectar, have been described for this plant (Carter and Thornburg, 2000, 2004b, c; Naqvi *et al.*, 2005). These proteins function in the nectar redox cycle, a biochemical pathway that produces high levels of hydrogen peroxide as an antimicrobial agent (Carter *et al.*, 2007). Ornamental tobacco nectarines are bright orange due to the accumulation of β -carotene (Horner *et al.*, 2007), which, together with ascorbic acid, provides the counter-balancing antioxidants needed to protect nectary cells, and probably the rest of the gynoeceum, from the oxidative environment caused by H_2O_2 . It was found that *Petunia hybrida* nectar is low in H_2O_2 levels and, further, that the addition of catalase has no effect on the antibacterial activity of petunia nectar. Thus, the strong antibacterial activity found in petunia nectar was not dependent on the accumulation of H_2O_2 .

It was also found that petunia nectar is rich in nuclease activities, in particular RNases, although DNases are also detected in this nectar. By contrast, while present, these enzymes are not detected at high levels in tobacco nectar. Differences in the patterns of RNase and DNase activities between these two plants are not limited to nectar. Other floral parts also show differential patterns, with enrichment in RNases in the 20–27 kDa range in petunia, and enrichment in activities probably corresponding to bifunctional nucleases in the 27–38 kDa range in tobacco. Increased levels of nuclease activities, both RNases and DNases, have been observed in many plants in response to bacterial, viral, and fungal pathogens (Lusso and Kuc, 1995; Floryszak-Wieczorek and Gniazdowska-Skoczek, 2001; Šindelářová and Šindelář, 2001; Kiba *et al.*, 2006), suggesting that these enzymes could have antimicrobial effects.

Nucleases are also involved in senescence and other programmed cell death processes (Dahiya, 2003). Thus, it is possible that some of the activities identified in our analysis are associated with senescence, which occurs rapidly for several floral tissues (O'Neill, 1997). This hypothesis, however, is not supported by the fact that most activities were found in anthers, the most RNase-rich tissue in flowers, before dehiscence. Thus, it is likely that at least some of these activities are performing biological functions not related to senescence.

Analyses of gene expression have identified two families of plant RNases as part of plant defence responses, pathogenesis related PR-10 proteins (Liu and Ekramodoullah, 2006), and S-like RNases (Bariola and Green, 1997). In this study, our attention was focused on the latter. Since S-like RNases have several highly-conserved amino acid motives, it was possible to amplify four petunia S-like RNases that had not been previously described. Two of those RNases, RNase Phy1 and RNase Phy5, were highly similar to well-characterized proteins from tobacco and tomato, respectively, and their expression patterns suggested that they may not be petunia nectarins. On the other hand, RNase Phy3 and RNase Phy4 were expressed in a pattern similar to that found for tobacco nectarins, suggesting that these enzymes may be part of the petunia nectar defence repertoire. Although only these two RNases are characterized here, they do not account for all the RNase activities found in petunia nectar (MS Hillwig, R Thornburg, GC MacIntosh, unpublished data).

S-like RNases have been implicated in defence responses against a variety of pathogens. Expression of the extracellular RNase NE from tobacco is induced by *Phytophthora parasitica* (Galiana *et al.*, 1997). Purified RNase NE inhibits hyphal growth from *P. parasitica* zoospores and from *Fusarium oxysporum* conidia *in vitro*, and co-infiltration of tobacco leaves with RNase NE and *P. parasitica* zoospores inhibited hyphal growth of the oomycete *in vivo* (Hugot *et al.*, 2002). While a direct antibacterial role for S-like RNases has not been demonstrated, expression of two rice S-like RNases is induced by *Xanthomonas oryzae* (MacIntosh *et al.*, 2010), and analysis of public microarray data indicates that *Arabidopsis* RNS1 and RNS2 are also induced by bacterial infections (data not shown). These data suggest that S-like RNases could have an antibacterial role. Expression of the related *RNase NGR3* and *RNase Nk1*, from different tobacco species, is also induced in response to tobacco mosaic virus and cucumber mosaic virus, respectively (Kurata *et al.*, 2002; Ohno and Ehara, 2005). In addition, *Arabidopsis* RNS1 is highly induced in response to mechanical damage both in local and systemic tissues (LeBrasseur *et al.*, 2002; Hillwig *et al.*, 2008). Tobacco RNase NW, Zinnia ZRNase II, and tomato RNase LE are also induced by wounding (Ye and Droste, 1996; Kariu *et al.*, 1998; Lers *et al.*, 1998). It has been suggested that the role of these secretory proteins during the wounding response is to block the spread of microorganisms that could penetrate through the wound site (LeBrasseur *et al.*, 2002).

The regulation of S-like RNases by varied pathogens and wounding suggests that these enzymes could have broad-spectrum antimicrobial activity that could be associated with cytotoxic properties of these proteins. In fact, it has been proposed that S-RNases involved in self-incompatibility probably evolved from S-like RNases that had a defensive role (Hiscock *et al.*, 1996; Nasrallah, 2005). S-RNases have a cytotoxic effect on the pollen tube during self-incompatible pollination. It is thought that, as the pollen tube elongates, the S-RNases are secreted into the extracellular matrix and may gain access into the cytoplasm of the pollen tube where they may degrade RNA from incompatible pollen (McClure and Franklin-Tong, 2006).

Secretory ribonucleases also have a defence role in animals. Several members of the vertebrate-specific RNase A family have antimicrobial properties. Human RNase 2 and RNase 3, two eosinophil associated RNases, have antiviral activity, and RNase 3 also has an antibacterial function. Angiogenin and RNase 7 have antibacterial and antifungal activities (reviewed in Boix and Nogues, 2007). Similarly, several zebrafish RNases, also members of the RNase A family, were shown to have antibacterial effect (Cho and Zhang, 2007). However, enzymatic activity is not essential for eosinophil associated RNases antimicrobial activity (Rosenberg, 1995; Torrent *et al.*, 2009). It has been proposed that their antimicrobial activity is due to the membrane destabilizing properties of these proteins. Positively charged amino acid residues in these proteins are thought to be important to disrupt negatively charged bacterial cell membranes and may be key to their bactericidal activity (Cho and Zhang, 2007, and references therein). Interestingly, while most S-like RNases are acidic proteins, RNase Phy3 and RNase Phy4 have high isoelectric points, indicating enrichment in basic amino acids. Thus, it is possible that the very basic nature of these proteins could indicate an antibacterial activity that can explain the effect on bacterial growth observed in our experiments.

In plants, RNase T2 proteins are divided in two classes, S-RNases and S-like RNases, based on biological role and phylogenetic relations (Igic and Kohn, 2001). However, some proteins do not fit this classification. Relic-RNases are RNases that are no longer associated with self-incompatibility, but they are clearly derived from S-RNases through gene duplication events (Golz *et al.*, 1998). Others, referred to as non-S RNases, seem to have intermediate characteristics between S-RNases and S-like RNases (Yamane *et al.*, 2003). RNase Phy3 and RNase Phy4 seem to fall into the latter category.

Both RNase Phy3 and RNase Phy4 are basic proteins, and RNase Phy3 has only one intron interrupting the coding region (M Hillwig, G MacIntosh, unpublished data). These are characteristics of S-RNases. However, the *RNase Phy4* gene is unusual because it does not have introns (M Hillwig, G MacIntosh, unpublished data). In addition, gene expression analyses showed that the expression pattern of RNase Phy4 (petals, ovaries, and nectaries) is very different from that of S-RNases, which are mainly expressed in pistils; *RNase Phy3* is also mainly expressed in ovaries and

nectaries, although in this case expression in stigma is also high. Analysis of the amino acid patterns present in both proteins also show that these proteins differ from both the canonical S- and S-like RNases, since RNase Phy3 has one of the two amino acid patterns characteristic of S-RNases, and one of the two patterns belonging to S-like RNases. RNase Phy4 only has one of the two S-like patterns, and none of the S-RNase patterns.

Yamane *et al.* (2003) identified a non-S RNase from *Prunus avium*, RNase PA1, that is also basic and has an expression pattern similar to S-RNases, but which has a low level of homology with this class of proteins; in addition, phylogenetic analyses placed RNase PA1 outside the S-RNase class. These authors proposed that this non-S RNase is a possible ancestral form of S-RNases. So far, this type of enzyme has been found only in other plants of the genus *Prunus* (Yamane *et al.*, 2003; Banovic *et al.*, 2009).

RNase Phy3 and RNase Phy4 do not have high sequence homology to the *Prunus* non-S RNases; however, they cluster together among class III RNases, and they share the intermediate nature between S- and S-like RNases based on amino acid patterns. Thus, these petunia proteins could be the Solanaceae equivalent of the *Prunus* enzymes, and represent an ancestral form of S-RNases.

Alternatively, these non-S RNases could represent relic S-RNases that lost their self-incompatibility function after gene duplication. *Petunia hybrida* possesses both functional and relic S-RNases proteins (Ai *et al.*, 1992; Lee *et al.*, 1992; Robbins *et al.*, 2000). However, it has been shown that relic S-RNases are always more closely related to the S-RNases from the same family than to other RNases (Golz *et al.*, 1998; Liang *et al.*, 2003). By contrast, petunia non-S RNases cluster with *Prunus* non-S RNases and other *Prunus* proteins, and there is some evidence that these non-S RNases are conserved in tobacco and tomato (M Hillwig, G MacIntosh, unpublished data). Thus, we favour the hypothesis that these non S-RNases are ancient, although a more detailed analysis of evolutionary relationships will be necessary to solve this question. The potential role of these enzymes as antimicrobial agents in nectar is consistent with the hypothesis that S-RNases were derived from enzymes involved in defence mechanisms against invading pathogens (Hiscock *et al.*, 1996; Nasrallah, 2005).

Although additional work may be needed to demonstrate an antibacterial role of RNase T2 enzymes in flowers, our work identifies for the first time the presence of these proteins in nectar. In addition, the large number of RNases and nucleases identified in other floral tissues indicates that these enzymes probably have additional roles in flowers.

Finally, the absence of hydrogen peroxide and the abundance of RNases in petunia nectar and the concomitant lack of these proteins in tobacco nectars support the hypothesis that nectar defences have evolved relatively recently.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Primers used in this work

Supplementary Fig. S1. Effect of tobacco and petunia nectar on bacterial growth.

Supplementary Fig. S2. Ribonuclease activities are present in nectar.

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