



Nectarin I is a novel, soluble germin-like protein expressed in the nectar of *Nicotiana* sp.

Clay Carter, Richard A. Graham and Robert W. Thornburg*

Department of Biochemistry and Biophysics, Iowa State University, 2212 Molecular Biology Building, Ames, IA 50011, USA (*author for correspondence)

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Abstract

We have identified a limited number of proteins secreted into the nectar of tobacco plants. Nectarin I is the most highly expressed nectar protein and has a monomer molecular mass of 29 kDa. The other major nectar proteins are expressed at lower levels and have monomer molecular masses of 41, 54, and 65 kDa respectively. Nectarin I was purified and antiserum was raised against the protein. Under nondenaturing conditions, Nectarin I has an apparent molecular mass of >120 kDa. The expression of Nectarin I was restricted to nectary tissues and to a much lower level in the ovary. No Nectarin I was found in petals, stems, leaves, or roots or other floral tissues. The expression of Nectarin I was also developmentally regulated. It is expressed in nectary tissues only while nectar is being actively secreted. Subsequently, the N-terminus of purified Nectarin I was sequenced. Sequence identity showed Nectarin I is related to wheat germin. Although hydrogen peroxide is readily detectable in tobacco floral nectar, we were unable to demonstrate any oxalate oxidase activity for Nectarin I. A partial cDNA encoding the mature Nectarin I N-terminus was isolated and used to probe a *Nicotiana plumbaginifolia* genomic library. The Nectarin I gene was isolated and the translated sequence was consistent with both N-terminal and internal cyanogen bromide-derived amino acid sequence. The gene contains a single 386 nt intron and encodes a mature protein of 197 amino acids.

Introduction

Many plants require insect or avian pollinators to obtain efficient seed set. Dicotyledonous plants often attract these pollinators with offerings of floral nectar that is secreted into the floral tube at the base of the ovary. Nectar is a rich source of sugars and amino acids and provides a reward to pollinators, thereby increasing the fecundity of those plants that provide nectar. The secretion of nectar is usually under developmental control beginning when the flowers open. After pollination, the nectar is frequently resorbed (Burquez and Corbet, 1991). In addition, nectar se-

cretion increases as the flower is visited by pollinators (Smith *et al.*, 1990).

The composition of nectar has been widely studied. Nectar is an aqueous combination of a significant number of substances. Chief among these are sucrose, glucose, and fructose. Other carbohydrates including arabinose, galactose, mannose, gentiobiose, lactose, maltose, melibiose, trehalose, melezitose, raffinose, and stachyose have also been identified in nectars of some flowers (Baker and Baker, 1981). Various types of nectars can be ordered into three groups according to sugar content: sucrose prevalent, glucose and fructose prevalent, and equal amounts of sucrose, glucose, and fructose (Roshchina and Roshchina, 1993). Interestingly, sugar concentrations vary greatly depending on the type and location of the nectary (Roshchina and Roshchina, 1993). A relationship exists between the sugar composition of the nectar and the amount of vascular tissue underlying the nectary (Esau, 1977). If

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phloem makes up most of the vascular tissue, the nectar may contain up to 50% sugar. On the other hand, if xylem predominates, the sugar content may fall to as little as 8% (Frey-Wyssling, 1955).

Some nectars also contain amino acids (Baker and Baker, 1973). All twenty of the normal amino acids found in protein have been identified in various nectars, with alanine, arginine, serine, proline, glycine, isoleucine, threonine, and valine being the most prevalent. Other substances reported in nectar include organic acids (Baker and Baker, 1975), terpenes (Ecroyd *et al.*, 1995), alkaloids (Deinzer *et al.*, 1977), flavonoids (Rodriguez-Arce and Diaz, 1992), glycosides (Roshchina and Roshchina, 1993), vitamins (Griebel and Hess, 1940), phenolics (Ferrerres *et al.*, 1996), and oils (Vogel, 1969). Using laser mass spectroscopic microanalysis, Heinrich (1989) found that the major cation of most nectars was K^+ , making up 35–74% of the total cation content. Averages of other notable cations were Na^+ (17.9%), Ca^{2+} (12.8%), Mg^{2+} (5.9%), Al^{3+} (4.6%), Fe^{3+} (1.2%), and Mn^{2+} (0.8%).

Notable among these analyses of the composition of nectar is the lack of studies characterizing the presence of proteins in nectar. Although the presence of proteins in nectar has been long reported (Beutler, 1935), there has not been to date a focused examination of the presence of proteins in nectar. Indeed other than secreted digestive enzymes in carnivorous plants (Scala *et al.*, 1969; Heslop-Harrison and Knox, 1971), only a single report (Peumans *et al.*, 1997) has characterized any nectar proteins.

In this report, we have determined that tobacco plants secrete a limited array of proteins into their nectar. Subsequently, we identified the major nectar protein, Nectarin I, as a novel germin-like protein (GLP). We have characterized its expression and have isolated the gene encoding Nectarin I.

Materials and methods

Plant material

The tobacco plants used in this study were derived from an interspecific cross between *Nicotiana glauca* and *N. sanderae*. Both of these species are diploid and belong to the *Alatae* section of *Nicotiana*. These plants were previously used to study a genetic instability (Kornaga *et al.*, 1997) that was initially observed in the first half of this century

(Smith, 1937) and later lost. The genetic instability within these plants behaves like a transposable element and was found to reside at a genetic locus that encodes flavonoid 3'-hydroxylase, dehydroflavonol reductase, leucodelphinidin dehydratase, or a regulatory locus controlling the expression of one of these loci (Kornaga *et al.*, 1997). Later, these plants were self-pollinated to produce a compact highly flowering, ornamental variety of tobacco (Kornaga and Thornburg, unpublished). The original Nectarin tests were done with plants from the F₄ generation. All tobacco plants were grown under 16 h day/8 h night in the greenhouse.

Nectar collection

Nectar was collected from tobacco plants by gently separating the floral tube from the calyx of the flower and squeezing the base of the floral tube. The droplet of nectar exuded from the collapsing floral tube base was collected in a microfuge tube. Each flower collected produced ca. 20 μ l of nectar. Nectar samples were kept on ice and routinely stored at $-20^{\circ}C$ prior to use. For enzymatic assays, nectar was used immediately following collection.

Enzymatic assays

The procedure described by Sugiura *et al.* (1979) was used for the assay of oxalate oxidase activity in solution, using a commercial preparation of barley oxalate oxidase as a positive control.

Cyanogen bromide digestion

Nectar (1 ml) was combined with 9 ml of ice-cold 100% ethanol in an Oak Ridge tube, mixed by inversion, and incubated on ice for 15 min. The solution was then centrifuged at $65\,000 \times g$ in a Ti70 rotor for 20 min. The pellet was resuspended in 100 μ l of 10 mM phosphate buffer, pH 7.4, and run on a 12% SDS-PAGE according to the method of Laemmli (1970). This ethanol precipitation method resulted in recovery of 60% of total nectar protein but only 5% of nectar carbohydrate. After electrophoresis, the gel was briefly stained in 0.5% Coomassie blue and destained until the protein bands became visible. The protein band corresponding to Nectarin I was excised from the gel with a razor blade and placed in a 10 ml beaker. The beaker containing the gel slice was then placed inside a 500 ml Erlenmeyer vacuum flask containing 20 mg of cyanogen bromide dissolved in 1 ml

of trifluoroacetic acid (Wang *et al.*, 1994). The flask was sealed with a rubber stopper, twice purged with nitrogen, and sealed under nitrogen in the dark.

After a 24 h incubation period, the gel slice was removed from the flask and subjected to many changes (about 30) of 4× loading buffer (Laemmli, 1970) until the solution remained blue for longer than 1 min. A 3 mm thick preparative 17% SDS polyacrylamide gel was prepared. The gel slice was loaded onto the new gel and electrophoresed for 8 h at 100 V. After electrophoresis, the gel was stained for 2 h in 0.1% Coomassie blue and destained overnight. The proteins were then transferred onto PVDF membranes by electroblotting. A major protein fragment with a molecular mass of ca. 15 kDa was identified and sequenced on an Applied Biosystems 477A protein sequencer/120A Analyzer using sequential Edman degradation at the Iowa State University protein facility.

DNA methods

Cloning methods were conducted either according to the manufacturer's directions or by accepted methods (Sambrook *et al.*, 1989). PCR was performed according to the methods outlined in Erlich (1989). Oligonucleotides used for PCR amplification of the Nectarin I cDNA are Nec I oligo1 (5'-GCNGAYGARGAYATGYT-3') and Nec I oligo2 (5'-RAARTCNGCNGCNGTRAA-3'), where R = G + A, Y = T + C, and N = G + A + T + C. The *Nicotiana plumbaginifolia* genomic library was kindly supplied by Dr Stephen Howell, Department of Biochemistry, Cornell University.

Nectarin I isolation and antiserum production

The Nectarin I protein was purified by preparative SDS-PAGE. Nectar (1 ml) was isolated from a single plant, denatured and electrophoresed on a 12% SDS polyacrylamide gel. After 3 h, the gel was briefly stained with Coomassie blue, destained and the protein band corresponding to Nectarin I was excised from the gel and lyophilized. The lyophilized gel was crushed and resuspended in 0.5 ml of phosphate-buffered saline. The homogenized gel (100 μ l) was homogenized with 1 ml of Freund's complete adjuvant for injection into a female New Zealand white rabbit. Boosts consisting of 100 μ l aliquots homogenized with 1 ml of Freund's incomplete adjuvant were given on days 15 and 30. Beginning on day 54, blood was withdrawn from the rabbit, clotted, and processed for antiserum.

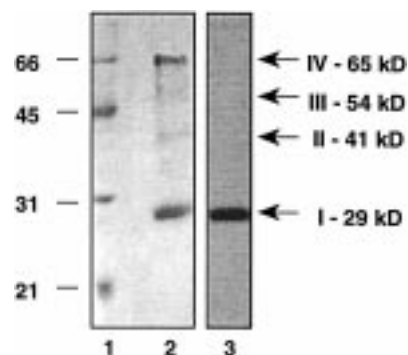


Figure 1. PAGE and western blot of nectar proteins. A 10% SDS polyacrylamide gel containing molecular weight marker proteins (lane 1) or 50 μ l of fresh nectar (lane 2) was run according to the methods of Laemmli (1970). The resulting gel was stained with Coomassie Blue and photographed. For western blots (lane 3) proteins were transferred onto nitrocellulose, blocked in Blotto (Sambrook *et al.*, 1989), reacted with anti-Nectarin I antiserum, and subsequently reacted with [125 I] protein A.

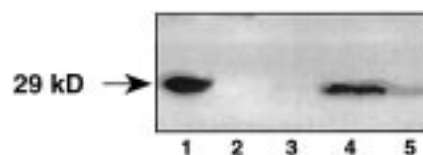


Figure 2. Western blot of tobacco flower tissues. Proteins were separated on a 10% SDS-PAGE and processed for western blotting as described in the legend to Figure 1. Each lane contained 100 μ g of total protein isolated from various floral tissues. Lane 1 contained boiled nectar, lane 2 boiled protein isolated from anthers, lane 3 from styles, lane 4 from nectaries, and lane 5 from ovaries.

SDS PAGE and western blotting

SDS-PAGE was conducted according to the methods of Laemmli (1970). Western blots were conducted according to the methods of Timmons and Dunbar (1990).

Results

We had previously bred a small, ornamental tobacco variety from an interspecific cross between *Nicotiana langsdorffii* and *N. sanderae* (Kornaga *et al.*, 1997). These plants are about 30 cm tall, yet they typically produce numerous (50 to 70) large inflorescences. Uniquely, these plants also produce copious quantities of nectar.

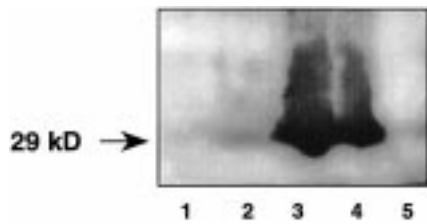


Figure 3. Nectarin I expression in developing nectary and ovary tissues. Proteins were separated on a 10% SDS-PAGE and processed for western blotting as described in the legend to Figure 1. Each lane contains 100 μg of total protein isolated from nectary and ovary tissues at different developmental flower stages. Lane 1, floral tube length 0.5 cm; lane 2, floral tube length 2 cm; lane 3, mature unopened flower, floral tube length 4 cm; lane 4, mature open flower, floral tube length 4 cm; lane 5, mature post-fertilization flower, floral tube length 4 cm.

Tobacco flowers secrete a limited array of proteins into nectar

We initially attempted to quantitate protein in nectar by the method of Bradford (1976). This analysis demonstrated the presence of ca. 240 $\mu\text{g}/\text{ml}$ of protein in the nectar. We then used SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to examine the nature of these proteins. This analysis verified that there indeed were proteins present in the nectar of these plants (Figure 1, lane 2). The major nectar protein, Nectarin I, has a molecular mass of 29 kDa when analyzed by SDS-PAGE; the three other major nectar proteins have molecular masses of 41, 54, and 65 kDa, respectively, and are present at lower levels than Nectarin I. The nectar protein patterns from different plants segregating from the original *N. langsdorffii* \times *N. sanderae* cross were identical.

To determine whether these proteins were immunologically related, we purified Nectarin I by preparative SDS-PAGE and raised antiserum against this protein. This antiserum was used to probe western blots of total nectar proteins (Figure 1, Lane 3). As can be observed, the Nectarin I protein is unique and the other nectar proteins do not react with the anti-Nectarin I antiserum. Preimmune antiserum showed no interactions with any nectar proteins.

Tissue-specific and developmental expression of Nectarin I

To evaluate the expression of the Nectarin I protein in different plant tissues, proteins were isolated from various flower tissues as described in Materials and methods. Equivalent amounts of the proteins from the various tissues were electrophoresed, transferred onto

nitrocellulose, and probed with anti-Nectarin I antiserum. The results, shown in Figure 2, indicate that the highest level of the Nectarin I protein in floral tissues was found in the nectary (lane 4), with a much lower level found in ovary tissues (lane 5).

In a separate experiment, proteins were isolated from petals, roots, stems, and leaves of tobacco plants. None of these tissues were found to contain proteins that reacted with the anti-Nectarin I antiserum (data not shown). Thus, it appears that high levels of Nectarin I expression were restricted within the nectaries and, to a much lower level, within the ovary.

We have also examined Nectarin I expression during nectary development. Nectary tissues develop early in flower primordia. By the time the flower is 0.5 cm long, there is already a dark green band of cells at the base of the ovary that will eventually develop into the nectary. When flowers are 2 cm long, the nectary color begins to turn a pale yellow. Nectarin I is not expressed at these early developmental stages, as can be seen in Figure 3, lanes 1 and 2. Just prior to flower opening, when the flower is about 4 cm long, the nectary turns bright orange and begins to swell. At this time, Nectarin I production begins (lane 3). The flowers remain open for three to five days while fertilization occurs. Nectar secretion and Nectarin I synthesis (lane 4) both actively occur while the flowers remain open. After fertilization, the flower petals wilt, the style drops from the tip of the ovary, and nectar production ceases. One week after fertilization, Nectarin I is no longer present in the nectary or ovary tissues (lane 5). As development continues, the ovary distends with developing seeds. Although no longer functional, a clearly discernible band of nectary tissue remains even after seed-pod dehiscence.

Identification of Nectarin I as a germin-like protein

To determine the biochemical identity of Nectarin I, we purified this protein by preparative SDS-PAGE, blotted it onto PVDF membrane and determined the N-terminal sequence at the Iowa State University protein facility. This sequence was determined to be ADEDMLQDVXVADLHSEKVKVNGFPPKAXFTAADFSSLAISKPGAT, where X is unknown. The sequence of the first 24 amino acids was high of quality, whereas the remainder was of lower quality. A Blast search (Altschul *et al.*, 1990) demonstrated that the Nectarin I N-terminal sequence was related to wheat germin. Figure 4 presents the alignments of

Nectarin I		ADEDMLQDVCVADLHSK	VKVNQFPCKA
Wheat Germin	MGYSKNLGAGLFGMLLLAPGVLA	SDPDPLQDFCVADLDGKAVSVNGHPCKP	
Barley Germin	MASSCSFLLLAALLALVSWQATS	SDPSPLQDFCVADMHSP	VRVNGFVCKN
Barley OxOxase		SDPDPLQDFCVADLDGKAVSVNGHTCKP	
Rhicadhesin Receptor		ADADALQDLQVADYASV	ILVNGFASKP

Figure 4. The purified Nectarin I protein was blotted onto PVDF membrane and the N-terminal sequence was determined at the Iowa State University protein facility. A Blast search (Altschul *et al.*, 1990) showed identity between Nectarin I and the class of proteins termed germins. The presented sequences are: the Nectarin I N-terminal sequence (this report) compared with the N-terminal sequences of wheat germin (GenBank accession number Y09917), barley germin (X93171), barley oxalate oxidase (P45850), and pea rhicadhesin receptor (S42642). The shaded areas represent amino acid sequence identity.

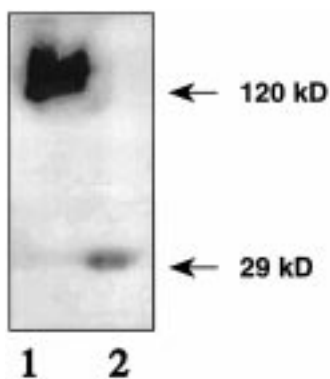


Figure 5. Western blot of boiled and unboiled nectar proteins. A 10% SDS-PAGE was run. The sample in lane 1 contained 50 μ l of nectar that was not denatured by boiling prior to loading on the gel. The sample in lane 2 was processed according to the method of Laemmli by boiling the 50 μ l nectar sample for 3 min prior to loading on the gel. Nectarin I proteins were visualized by western blotting.

the Nectarin I N-terminal sequence with other related proteins.

Germins are apoplastic proteins (Lane *et al.*, 1986) that were first identified in imbibing wheat embryos (Thompson and Lane, 1980). They are ca. 30 kDa proteins that form either pentamers (McCubbin *et al.*, 1987) or trimer/dimers (hexamers) (Woo *et al.*, 1998). They are glycoproteins and they also adventitiously bind large amounts of cell wall fragments (Jaikaran *et al.*, 1990). A closely related but distinct isoform of germin termed pseudogermin has been shown to accumulate in developing wheat seeds. The N-terminal sequences of the germin and pseudogermin are very similar, but pseudogermin has unique thermal stability. It is able to maintain its protein structure at 100 °C ($t_{1/2} = 6$ min; Lane *et al.*, 1992).

Recently an enzymatic activity was identified for wheat germin. Lane *et al.* (1993) demonstrated that wheat germin also functions as an oxalate oxidase. This protein produces hydrogen peroxide by oxidation of oxalic acid. We attempted to determine whether Nectarin I showed any oxalate oxidase activity. Al-

though hydrogen peroxide could be readily detected in floral nectar, we were repeatedly unable to demonstrate conclusively that Nectarin I had oxalate oxidase activity. Therefore, it appears that Nectarin I is structurally related to oxalate oxidase.

As stated previously, another unique aspect of germins and especially pseudogermin (Lane *et al.*, 1992) is that they have an unusually stable quaternary structure. To evaluate the quaternary structure of Nectarin I, we ran SDS-PAGE analyses of boiled and unboiled nectar. We used western blots to visualize the Nectarin I protein. As can be seen from Figure 5 (lane 2), in boiled nectar, Nectarin I runs as a 29 kDa monomer, whereas in unboiled nectar (lane 1), the Nectarin I protein runs as a diffuse high-molecular-mass band of >120 kDa. This size is consistent with both the pentamer model (McCubbin *et al.*, 1987) and the trimer/dimer model (Woo *et al.*, 1998) for germin-like proteins. Germin is also known to adventitiously bind large amounts of cell-wall fragments (Jaikaran *et al.*, 1990). The diffuse character of the undenatured Nectarin I protein observed in Figure 5 is also consistent with carbohydrate binding to the Nectarin I protein.

Isolation and characterization of the Nectarin I gene

Based upon the amino acid sequence of the Nectarin I N-terminal peptide, we constructed a pair of degenerate oligonucleotides for PCR amplification of the Nectarin I mRNA. Sequences of these oligonucleotides are presented in Materials and methods. Messenger RNA was isolated from the nectaries of the Nectarin I-producing plants. The first-strand synthesis was performed by reverse transcription. After the first-strand synthesis, PCR was used to generate a partial cDNA fragment. The fragment was blunt-end subcloned into the *HincII* site of pUC19 to generate the clone pRT448. This partial cDNA was 102 nucleotides in length and contained amino acids 1 to 34 of the N-terminal sequence. The sequence of the insert is presented in Figure 6 along with the amino acid translation of pRT448 compared with the N-

terminal sequence of the Nectarin I protein. Only a single amino acid difference was identified between the N-terminal sequence and the translated amino acid sequence of the cDNA (underlined in Figure 6).

This partial Nectarin I cDNA clone was used to screen a *Nicotiana plumbaginifolia* genomic library. Positive plaques were taken through three rounds of screening, which yielded a positive clone called lambda *Np*-Nectarin I. The gene was mapped using the pRT448 insert, and a 3662 nucleotide *Bgl*III fragment was subcloned and sequenced. This clone, pRT454, contained the full-length Nectarin I gene. The Nectarin I sequence was deposited in GenBank as accession number AF132671.

To confirm that this isolated Nectarin I gene does indeed encode the Nectarin I protein, we performed cyanogen bromide cleavage of the mature Nectarin I protein as described in Materials and methods. Subsequently, we isolated the major cleavage fragment and determined the amino acid sequence of this peptide. Nine amino acids were identified from this analysis (MEGELDVGF). This sequence is indeed found within the Nectarin I gene at codons 129 to 137 of the preNectarin I protein. Thus, not only does the N-terminus of the mature protein match the Nectarin I gene, but the internal amino acid sequence also matches the gene sequences thereby confirming that this gene encodes the Nectarin I protein.

The Nectarin I protein is encoded by two exons separated by a single 386 bp intron. The intron interrupts an (A[^]AG) codon Lys-49 of the preNectarin I protein. We compared the structure of the tobacco Nectarin I gene with structures of known *Arabidopsis* GLP genes: GLP1 (Carter and Thornburg, 1998), GLP2b, GLP9 (Carter *et al.*, 1998), and GLP5, GLP7, GLP8, GLP10 (Carter and Thornburg, unpublished). In the case of GLP1, GLP3b, and also with wheat germin (Figure 7), the GLP genes are encoded as a single exon; however, for most *Arabidopsis* GLP genes the two exons/one intron pattern is typical. Interestingly, the tobacco intron is located at the same exact nucleotide as those introns found within the *Arabidopsis* GLP5 and GLP10 genes. As shown in Figure 8, these are the *Arabidopsis* genes that are most closely related to the Nectarin I gene. In those *Arabidopsis* genes that do contain introns, the average intron length is 94 nt (84 to 107 nt) in contrast to the longer, 386 nt, tobacco intron.

We also examined the 1.3 kb upstream promoter portion of the gene that regulates its expression. Initially we identified a putative TATA box that shows

high similarity to the consensus plant TATA sequence (Joshi, 1987). This sequence is located 90 nt upstream from the ATG start codon. A 'CCAAT' sequence is located 29 nt upstream from the TATA box. Wheat germins are known to be regulated by auxin (Berna and Bernier, 1997). Both wheat germin genes (Berna and Bernier, 1997) and *Arabidopsis* GLP genes (Carter and Thornburg, 1998; Carter *et al.*, 1998) contain auxin regulatory sequences. Similarly, the Nectarin I gene contains two potential auxin regulatory sequences that are similar to the Auxin A Box defined by McClure *et al.* (1989). Furthermore, four bZip protein-binding sites (Schmidt, 1993) are located throughout the 1.3 kb promoter. To understand whether these sequences function to regulate the expression of the Nectarin I gene will require further experimentation.

Characterization of the Nectarin I protein

The Nectarin I protein translated from the Nectarin I gene was examined using several on-line DNA analysis tools. The PSORT analysis tool (Nakai and Kanehisa, 1992) predicts the presence of N-terminal signal sequences based upon the methods of McGeoch (1985) and von Heijne (1986). The results of this analysis predicted the presence of a 32 amino acid N-terminal signal sequence with the mature protein beginning with the sequence ADEDMLQ.... This is the exact sequence verified by our N-terminal sequencing of the mature Nectarin I protein. Therefore, the preNectarin I protein is translated as a 229 amino acid protein with a molecular mass of 24 765 Da and a pI of 7.71. After removal of the 32 amino acid signal sequence the resulting mature Nectarin I has a molecular mass of 21 062 Da and a pI of 7.12. The PSORT tool also predicts the subcellular localization of proteins. The Nectarin I protein was predicted with relatively high certainty (78.6%) to be expressed extracellularly, as indeed it is found to be.

A single site of N-glycosylation was identified (Asn-60). Interestingly, all *Arabidopsis* GLPs and all wheat and barley germins have a glycosylation site in a similar location. Structurally this glycosylation site exists in a predicted loop region (Carter *et al.*, 1998; Gane *et al.*, 1998).

Phylogenetic analysis of Nectarin I, germins, and GLPs

To examine the phylogenetic relationships of the Nectarin I protein with germins and other germin-like

GLP subfamilies. As in *Arabidopsis*, the largest subfamily is subfamily 1. Nectarin I belongs to subfamily 2. The non-*Arabidopsis* GLPs that are most closely related to Nectarin I are the germin-like proteins from *Solanum tuberosum* and *Lycopersicon esculentum*. To determine whether these proteins are expressed in the nectar of solanaceous plants or of *Arabidopsis* will require further analysis.

Discussion

Although the presence of protein in nectar has long been reported, the characterization of nectar proteins has lagged far behind. With the exception of the work in this report, to our knowledge only one other report (Peumans *et al.*, 1997) has examined proteins in floral nectars. In that study, the authors identified two proteins, alliin lyase and mannose-binding lectin, that are present in the nectar of leek flowers.

We have examined tobacco nectars and determined that these plants secrete a limited array of proteins in their nectar. The most highly expressed protein, Nectarin I, was purified and antiserum was raised. Western blot analyses permitted us to evaluate its spatial and temporal expression. Nectarin I was not detected in tissues other than the nectary and to a much lower extent in the ovary. Temporal analysis demonstrated that Nectarin I expression coincided with nectar secretion. Thus, there is an apparent coordination between the secretion of nectar from the nectary and Nectarin I expression.

N-terminal analysis of Nectarin I indicates that this protein belongs to a family of proteins termed germin-like proteins. Germin is a 130 kDa protein that was first detected in germinating cereals (McCubbin *et al.*, 1987). The extensive nature of this large protein family has only become apparent within the past few years. Germin-like proteins (GLP) have been identified in all species (monocot, dicot and gymnosperm) that have been examined to date. The best characterized germin-like protein family is in *Arabidopsis*, where there are at least 27 individual family members (Membré *et al.*, 1997; Carter *et al.*, 1998; Sato *et al.*, 1998). Thirteen of these have recently been identified in a locus that is present on three overlapping BAC clones (K15E6, MXF12, and K3K3) on chromosome V (Domon *et al.*, 1995; Sato *et al.*, 1998). The GLP proteins are expressed in a variety of tissues including seedlings, shoots, and immature siliques, but no analysis has been performed to examine expression in *Arabidopsis*

flowers or nectaries. All of these GLPs are structurally related (Carter *et al.*, 1998), yet their true function remains unknown. The simple fact that the *Arabidopsis* genome contains 27 genes encoding GLPs suggests that the function of these proteins must be important.

A variety of functions have been proposed for germins and GLPs, including secondary hydration of germinating embryos (Lane, 1991), responses to osmotic stress (Lane *et al.*, 1992), and chaperones of cell wall oligosaccharides (Jaikaran *et al.*, 1990). Based upon the recent identification of wheat germin as an oxalate oxidase, these proteins have been proposed to provide hydrogen peroxide that could participate in cell wall crosslinking (Lane *et al.*, 1993) or in plant defense (Levine *et al.*, 1994). Recently, several groups have demonstrated that both germin and oxalate oxidase activity increased during powdery mildew attack of wheat and barley (Dumas *et al.*, 1995; Zhang *et al.*, 1995; Hurkman and Tanaka, 1996) thereby strengthening the proposal that these proteins may be involved in plant defense.

As shown in Figure 4, another interesting protein identified by the Nectarin I N-terminal sequence was the plant receptor for rhicadhesin (Swart *et al.*, 1994). Rhicadhesin is a protein made by *Rhizobium* that mediates the initial stages of attachment and binding of these bacteria to plant cells (Smit *et al.*, 1989). The rhicadhesin receptor is a germin-like protein, closely related to Nectarin I, that was purified from pea roots based upon interactions with rhicadhesin (Swart *et al.*, 1994). The isolation of this protein demonstrates that bacteria do interact with plant cells through germin-like proteins. While the primary function of germin-like proteins remains unknown, bacterial adhesion should be teleologically disfavored. Thus, the bridge between bacterial and plant cells may be an evolutionarily acquired function of bacterial adhesins similar to that of bacteriophage Lambda tail protein, *gpJ*, that interacts with the maltose transport protein (Randall-Hazelbauer and Schwartz, 1973).

Nectar is not sterile. Insect or avian pollinators certainly transfer microorganisms from flower to flower. Based upon the fact that germin-like proteins interact with bacterial adhesins, a soluble germin-like protein (Nectarin I) could bind these adhesion proteins in solution thereby interdicting any bacterial-plant interactions that would be mediated by a rhicadhesin-germin-like protein system.

Swart *et al.* (1994) demonstrated that the interaction between rhicadhesin and the germin-like protein occurs at an Arg-Gly-Asp (RGD) tripeptide motif

within the germin-like protein. Nectarin I contains a variant of the RGD motif. Amino acids 119 to 121 of the mature protein are Lys-Gly-Glu (KGE). This motif is in the same location as the RGD tripeptide that occurs in other germin-like proteins.

The gene encoding the Nectarin I protein is structurally similar to other GLP genes that have been isolated. Most of these genes contain a single intron interrupting codon 16 to 20 of the mature protein. The Nectarin I intron interrupts codon 17 in the exact location as is found in the most closely related *Arabidopsis* genes, GLP5 and GLP10. To further characterize the nature of the nectary-specific gene expression found with the Nectarin I protein, we are currently analyzing the Nectarin I promoter and secretory sequences in transgenic plants.

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