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A major function of the tobacco floral nectary is defense against microbial attack

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Abstract. We have characterized the major nectar protein (Nectarin I) from ornamental tobacco as a superoxide dismutase that functions to generate high levels of hydrogen peroxide in nectar. Other nectar functions include an anti-polygalacturonase activity that may be due to a polygalacturonase inhibiting protein (PGIP). We also examined the expression of defense related genes in the nectary gland by two independent methods. We isolated a sample of nectary-expressed cDNAs and found that 21% of these cDNAs were defense related clones. Finally, we examined the expression of a number of specific defense-related genes by hybridization to specific cDNAs. These results demonstrated that a number of specific defense genes were more strongly expressed in the floral nectary than in the foliage. Taken together these results indicate that the floral nectary gland can have specific functions in plant defense.

Key words: Flower, gynoecium, nectar, nectary, PGIP, plant defense, nectar.

Introduction

Many plants require insect or avian pollinators to obtain efficient seed set. These plants attract these pollinators with offerings of floral nectar secreted into the floral tube at the base of the ovary. Nectar provides a reward to pollinators, thereby increasing the fecundity of the nectar-producing plants. The secretion of nectar is usually under developmental control beginning when the flowers open. Nectar secretion increases as the flower is visited by pollinators (Smith et al. 1990). After pollination, the nectar is frequently resorbed (Burquez and Corbet 1991).

Nectar is a mostly aqueous combination of a number of substances (Baker and Baker 1973a, b, Baker and Baker 1981). 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 almost 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).

Some nectars also contain amino acids (Baker and Baker 1973a). All twenty of the normal amino acids have been identified in various nectars, and alanine, arginine, serine, proline, glycine, isoleucine, threonine, and valine seem to be 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 (Ferreres et al. 1996, Cabras et al. 1999), and oils (Vogel 1969). Using laser mass-spectroscopic microanalysis, Heinrich (Heinrich 1989) found that the major cation in most nectars was K^+, making up 35 to 74 percent of the total cation content.

The importance of understanding the biology of nectar becomes apparent after considering that nearly half of the world's diet of fats and oils comes directly from insect-pollinated plants or from self-pollinated species that show increased pollination with insects; e.g. coconuts, cotton, oil palm, olives, peanuts, rape, soybeans and sunflower (Guidry 1964).

Materials and methods

Plant materials The nectar over-producing line of tobacco used in these studies was derived from an interspecific cross of *Nicotiana langsdorffii* Weinm. X *N. sanderae* Hort. Var Sutton's Scarlett and was previously described (Kornaga et al. 1997). *N. langsdorffii* is a valid taxonomic species native to southern Brazil. *N. sanderae* is a commercial adaptation of the flower color genes from *N. forgetiana* ex. Hemsl. (n = 9) in *N. alata* Lk. and Otto (n = 9) (East 1916, Smith 1937, Goodspeed and Thompson 1945, Sand 1957). The conditions for growth and the methods for collecting and processing nectar were previously described (Carter et al. 1999).

Microscopy Mature flowers (stage 12) were dissected to reveal circular nectaries at the base of the gynoecia. Some of these gynoecia were fixed with formalin-acetic acid-alcohol (FAA, Sass 1958), dehydrated in an ethanol series to 100% ethanol, critical point dried, mounted on aluminum stubs, and sputter coated with gold-palladium. Whole unfixed gynoecia with nectaries were viewed and imaged with an Olympus stereomicroscope fitted with a Zeiss Axiocam digital camera. The fixed nectary regions with guard cells were viewed and imaged with a JEOL 5800 digital scanning electron microscope at 10 kV. All images were processed using Adobe PhotoShop and PageMaker.

Polygalacturonase Inhibitor Protein (PGIP) assay Polygalacturonase (PG) was purified from *Botrytis cinerea* Pers. strain Del11 as described (Stotz et al. 1994). A plate assay for PGIP was performed essentially as described (Taylor and Secor 1988).

Array-based northern blot analysis Total RNA was isolated from the leaves and from developing (Stage 6) and mature (Stage 12) floral nectaries from ornamental tobacco plants. First strand cDNA was labeled by synthesis in the presence of [^{32}P]-dCTP. Radiolabeled cDNAs were then incubated with membrane filters containing tomato cDNAs representing control and defense genes that had been arrayed on the nitrocellulose filters. After hybridization and washing, the amount of bound radioactivity to each arrayed cDNA was quantified by PhosphorImager.

Results

In ornamental tobacco, nectar is secreted from a torus-shaped nectary at the base of the gynoecium (Fig. 1A), that has two vertically oriented special guard-cell zones (Fig. 1A; rectangle) spatially oriented 180° from each other. Each zone (Fig. 1B) contains special guard cells that appear occluded (Fig. 1C), partially occluded (Fig. 1D), or clear (Fig. 1E). The secreted nectar bathes the gynoecium. The rich composition of nectar and the non-sterile nature of visiting pollinators could easily lead to infection of the gynoecium.

Nectar proteins Prior to 1995, it had not been conclusively demonstrated that plant nectars contained a significant amount of protein. However, even from our earliest studies, it was clear that tobacco nectar did indeed contain proteins (Fig. 2). Using ornamental tobacco, we identified a total of five proteins

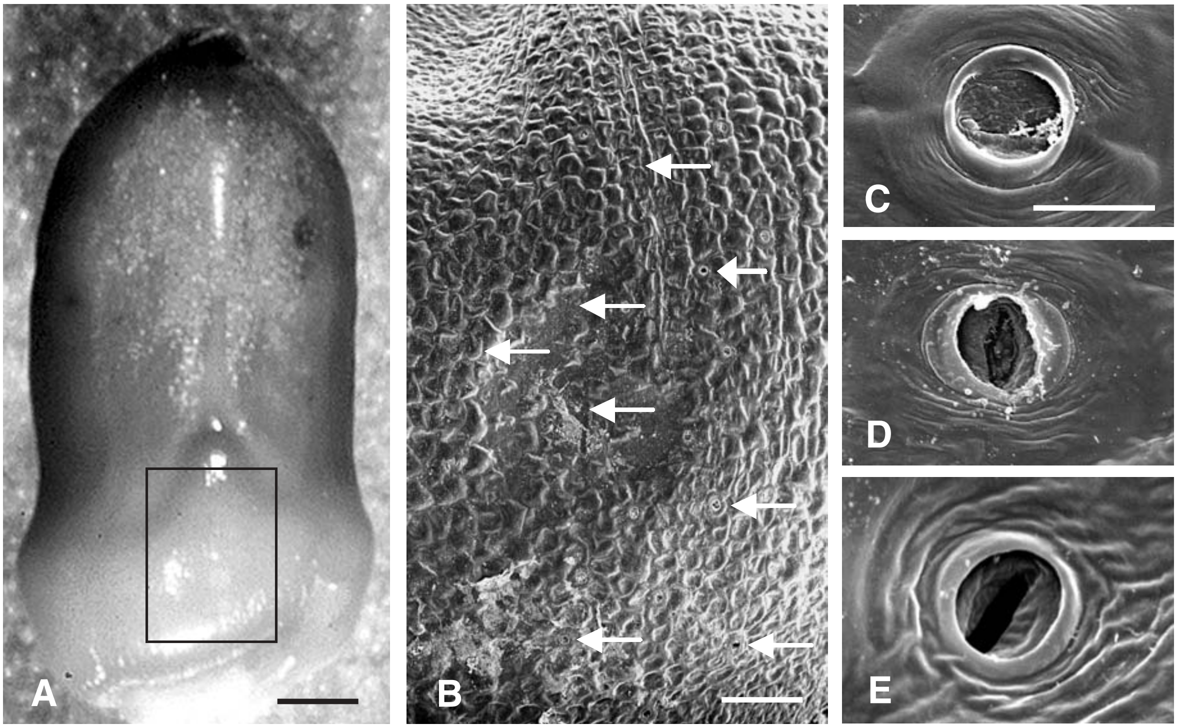

Fig. 1. Tobacco gynoecium, basal nectary and special guard cells (stage 12). **A** Exposed gynoecium showing a circular nectary at its base. Vertical rectangle delineates one of two zones (second at 180°, backside) containing special guard cells. Bar = 400 μm. **B** One zone showing special guard cells (arrows). Bar = 100 μm. **C–E** Special guard cells: occluded; partially occluded; clear. Bar = 10 μm

that accumulate in nectar to a total of 250 μg/ml. In our earliest studies, the most abundant protein, Nectarin I, was characterized as a germin-like protein (Carter, et al. 1999). Nectarin I was purified and was shown to be as a unique, manganese superoxide dismutase (SOD) that functioned to generate H_2O_2 (Carter and Thornburg 2000). We additionally showed that H_2O_2 accumulates to very high levels, up to 4 *millimolar*. This level of hydrogen peroxide is toxic to microorganisms (Carter and Thornburg, manuscript in preparation).

Nectar contains PGIP activity Another function of nectar proteins that we have identified is polygalacturonase inhibitor protein (PGIP). Plant cell walls are rich in pectin polysaccharides, polymers that contain galacturonic acid. The simplest of these pectins are the homogalacturonans, polymers whose backbone are cleaved by enzymes such as polygalacturonases (PGs) and pectate lyases. However some fungi have evolved a strategy to colonize plant tissues by expressing PGs. Fungal PG action permits pathogen entrance into plant tissues. To counter this activity, many plants have developed inhibitors of the fungal PGs (Brown and Adikaram 1983, Cervone et al. 1987, Stotz et al. 1993, Desiderio et al. 1997, Powell et al. 2000) which block the breakdown of plant cell walls by the fungus.

To evaluate the expression of PGIP-like proteins in nectar, we performed inhibition studies shown in Fig. 3. As a positive control the *Botrytis cinerea* polygalacturonase alone in sodium acetate buffer alone (spot 1) shows a large cleared zone of hydrolyzed polygalacturonic acid. When crude nectar is preincubated with the PG prior to assay, we observed a >90% reduction in the PG activity (spot 2). Similar observations with ammonium sulfate precipitated nectar proteins (spot 3) or with dialyzed nectar proteins (spot 4) indicate that the component causing the

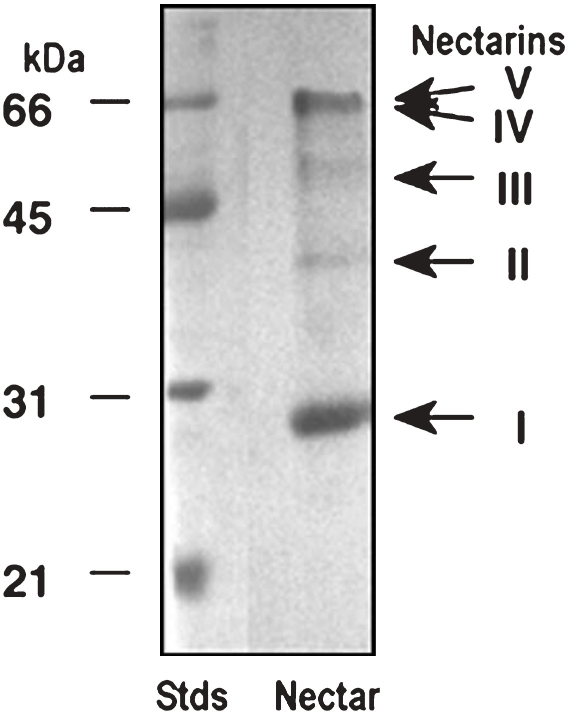

Fig. 2. SDS PAGE profile of nectar proteins from ornamental tobacco. Lane 1, molecular weight markers, Lane 2, 100 μl of raw nectar processed according to the methods of Laemmli (Laemmli 1970)

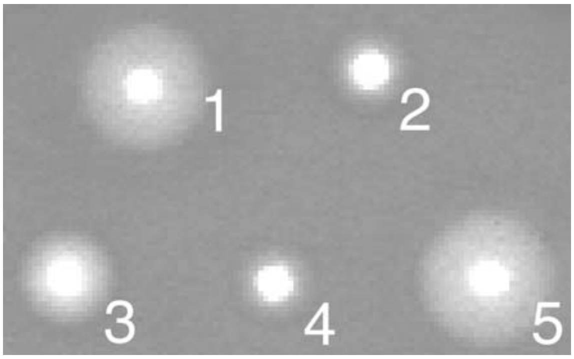

Fig. 3. PGIP activity in nectar. 15 μL of *Botrytis cinerea* PG was incubated either alone (spot 1) or with 15 μL of the indicated solutions and assayed for PGIP activity as outlined in Materials and Methods. Crude nectar (spot 2) was freshly isolated from tobacco flowers. Precipitated nectar proteins (spot 3) were prepared by an 87% ammonium sulfate fraction which was dialyzed against 50 mM sodium phosphate pH 7.0. Dialyzed nectar (spot 4) was prepared by dialyzing 500 mL of crude necter against 50 mM potassium phosphate pH 6.0/50 mM NaCl. Boiled nectar (spot 5) was prepared by boiling 100 μL of crude nectar for 5 minutes followed by immediate incubation on ice for 5 minutes

inhibiting activity behaves like a protein (i.e. it can be precipitated with ammonium sulfate and is larger than 10,000 Da in size). Further, we observed that boiling destroys the inhibitory activity found in nectar (spot 5) leading us to conclude that this activity is likely due to a nectar protein.

Preliminary analysis of nectary ESTs To evaluate the expression of different genes in nectary tissues, we have recently begun a limited EST analysis. We prepared a cDNA library from mature (Stage 12) nectary tissue of our ornamental tobacco and 60 ESTs were isolated for preliminary characterization to evaluate our libraries. Some of these defense genes are presented in Table 1. These genes include Nectarin I, the most abundant nectar protein, a number of metalothioniens whose function is something of an enigma, but appear to be involved in responses to metal stresses and homeostasis (Cobbett and Goldsbrough 2002); the antifungal flower-specific thionin (Gu et al. 1992), and the antibacterial peptide, Snakin 1 (Segura et al. 1999).

A preliminary analysis of our sequenced nectary ESTs reveals that 21% of the ESTs isolated encode defense-related gene products. This level of defense-related gene expression is quite high. Indeed other EST studies have shown that a more normal level of defense-related genes is about 6% of ESTs (Asamizu et al. 2000, Kim et al. 2001).

Array-based analysis of defense genes To gain a better insight into the expression of defense genes in tobacco nectaries, we have evaluated the levels of specific defense genes in leaves, and in developing (Stage 6) and mature (Stage 12) tobacco floral nectaries. To do this we have used an array-based Northern Blot analysis method. cDNAs were prepared from tobacco leaves and from two developmental stages of floral nectaries. These cDNAs were radiolabeled and hybridized to membranes arrayed with a number of tomato defensive

Table 1. Some tobacco floral nectary ESTs identified

Clone ID	Identity	Homolog
1A05	Nectarin I	AF132671
1A10	Wound-induced Sn1	S65081
1A11	Type 1 metalothionien	NGU46543
1A12	Late embyrogenesis abundant protein 5	AF053076
1C06	Snakin 1 antimicrobial peptide	CAC44032
1C11	Metalothionien-like protein	AJ223405
1D05	Type 2 metalothionien	U46543
1D12	Flower specific thionin	Z11748

genes cDNAs. The amount of hybridization to each spot was evaluated for leaves, developing and mature floral nectaries. The level of gene expression in the leaves was arbitrarily taken as 100% and the amount of expression in nectaries was normalized to that level. These data (Table 2) show, first of all, that there are a number of genes that do not significantly change in either Stage 6 or Stage 12 nectaries over the levels found in the leaf controls. Second, we observe a number of defense genes that are strongly up-regulated in the nectary relative to the leaf.

Discussion

In this study we present four lines of evidence that nectaries have a role in plant defense. First of all, we have previously demonstrated that nectar proteins are involved in the production of hydrogen peroxide (Carter and Thornburg 2000) that accumulates to very high levels in plant floral nectar (up to 4 *millimolar*). This is 40 times the level produced by human neutrophils in response to microbe attack (Prince and Gunson 1987). These levels are toxic to microorganisms (Carter and Thornburg, manuscript in preparation).

Second, we have demonstrated that the nectar of ornamental tobacco contains an active compound that functions to inhibit *Botrytis cinerea* PG. This compound may be a PGIP-like protein because its size is greater than 10,000 Daltons, its inhibitory activity is destroyed by boiling. PGIPs are often extracellular proteins and are expressed constitutively from a number of plants including tomato (Stotz et al. 1994), apple (Yao et al. 1999), and

Table 2. Expression of some floral nectary genes

Unchanged Genbank #	Enzyme	Stage 6		Stage 12	
		% of Leaf	SE	% of Leaf	SE
AI490845	Ser hydroxymethyltransferase	97.9	34.8	89.3	25.1
AI484386	Ribosome recycling factor	57.3	20.5	102.5	13.9
S72452	CBP20	74.3	21.4	108.9	30.9
Defense Genes Induced in Nectaries					
AW220441	PR-1	419.2	39.8	192.0	101.3
AW622107	PR-5	2454.3	359.0	214.2	118.6
AW220759	Chalcone synthase	451.9	128.2	210.6	100.2
AI490686	Wound-induced *win1*	379.6	48.4	482.5	175.5
AW092750	Wound-induced *pinI*	729.0	126.9	749.2	144.8
AI482620	Wound-induced *pinII*	439.3	49.4	335.4	28.5

pear (Stotz et al. 1993). In beans (Toubart et al. 1992) PGIPs can be induced by pathogens. We are currently evaluating potential PGIP proteins in the Solanaceae and Rosaceae.

Third, we have identified a number of plant defense proteins that are encoded by cDNAs expressed in developing and mature floral nectaries. These include a number of genes directly involved in antimicrobial and/or antifungal activities. Although our sample size is still small (60 clones), the finding that such a large proportion of these cDNAs encode defense-related genes implies that plant defense is an important function of the nectary.

Finally, we have used modified Northern Blot hybridization to demonstrate that mRNAs encoding a number of defense proteins are up regulated in the nectary gland over the levels found in foliage. The up-regulated defense genes include several homologues of tomato and potato wound-inducible genes including the proteinase inhibitor genes, *pin1* and *pin2* (Green and Ryan 1972, Ryan 1981), *win1* (Ponstein et al. 1994), and the antifungal pathogenesis-related proteins PR1 and PR5 (Stintzi et al. 1993).

Based upon these lines of investigation we are led to the conclusion that a major but heretofore unrecognized function of the nectary gland is in plant defense to protect the gynoecium from microbial attack. The mechanisms involved in this protection include induction of a number of mRNAs encoding defense-related proteins in the nectary gland itself and the expression of defense-related factors that are secreted into nectar.

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