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Is the nectar redox cycle a floral defense against microbial attack?

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Many angiosperms use a remarkable reproductive strategy that relies on attracting animals (insect, avian or mammalian pollinators) to transfer pollen between plants. Relying on other organisms for sexual reproduction seems evolutionarily untenable, but the great diversity of angiosperms illustrates how highly successful this strategy is. To attract pollinators, plants offer a variety of rewards. Perhaps the primary floral reward is floral nectar. Plant nectar has long been considered a simple sugar solution but recent work has demonstrated that nectar is a complex biological fluid containing significant and important biochemistry with the potential function of inhibiting microbial growth. These results lead the way to novel insights into the mechanisms of floral defense and the co-evolution of angiosperms and their pollinators.

Nectar is a metabolically rich fluid produced by an often distinct, terminally differentiated floral organ termed the nectary or sometimes, the nectary gland. In ornamental tobacco, the secretion of floral nectar from the nectary is under specific developmental control. Secretion begins when the flower opens and continues while the flower remains receptive to pollination. Nectar flow is often increased by pollinator visitation [1]. However, after pollination, nectar secretion ceases and the nectar remaining in the flower is frequently reabsorbed [2].

Floral nectar is usually an aqueous combination of a significant number of solutes. Chief among these are sucrose, glucose and fructose [3], but at least 11 other carbohydrates have been identified in floral nectars [4]. Most nectars also contain amino acids. All 20 of the normal amino acids found in protein have been identified in various nectars [5]. Other substances reported to occur in nectar include organic acids, terpenes, alkaloids, flavonoids, glycosides, vitamins, phenolics and oils [3,6–13]. The major cation in most nectars is potassium (35–74%) whereas ferric iron represents 2–3% of the total cation content [14]. Thus, floral nectar is a surprisingly rich medium with many components required for cellular growth and metabolism. There is little wonder that visiting pollinators choose to collect such free offerings. However, pollinators are not sterile and airborne pathogens can also be introduced into nectar. Considering it is surrounded by such a rich medium in which microorganisms could grow, it is surprising that there are so few reported infections of the gynoecium.

In addition to the other nectar components, many angiosperms also secrete a limited array of proteins termed nectarins into nectar [15]. Although the presence of proteins in plant nectars has been described historically [16–20], other than the secreted digestive enzymes of carnivorous plants, [21–23], only a single report [24] has previously characterized any biological activity associated with any nectar proteins. A list of proteins known to be secreted into nectar along with their presumed functions is presented in Table 1. This review will focus on three of the nectarin proteins that are present in the nectar of ornamental tobacco.

Nectarins: proteins accumulating in plant nectars

We have shown that ornamental tobacco plants secrete a specific but limited array of five proteins into their nectar (Figure 1). These proteins were named Nectarin I (NEC1) through Nectarin V (NEC5) in order of increasing molecular mass. In ornamental tobacco, nectarins accumulate to almost 250 μg/ml in nectar [15]. Understanding the role that these proteins play in nectar has identified a novel biochemical pathway that generates high levels of hydrogen peroxide yet permits the plant to deal with these high levels. We propose, that the primary function of this biochemistry is to defend the floral reproductive tract from contamination by microbes brought to the flower by nonsterile pollinators or by airborne means.

Nectarin I

The most abundant protein accumulating in the nectar of ornamental tobacco is Nectarin I (NEC1). This protein represents ~50% of total nectar proteins and SDS PAGE

Table 1. Proteins identified in the nectars of plants

Nectar protein	Species	Function	Refs
Alliin lyase	*Allium porrum*	Plant defense	[24]
Mannose lectin	*Allium porrum*	Plant defense	[24]
Nectarin I (NEC1)	*Nicotiana* spp.	SOD	[25]
Nectarin III (NEC3)	*Nicotiana* spp.	CA MDA reductase	[43]
Nectarin V (NEC5)	*Nicotiana* spp.	Glucose oxidase DHA reductase	[42]

Abbreviations: CA, carbonic anhydrase; SOD, superoxide dismutase.

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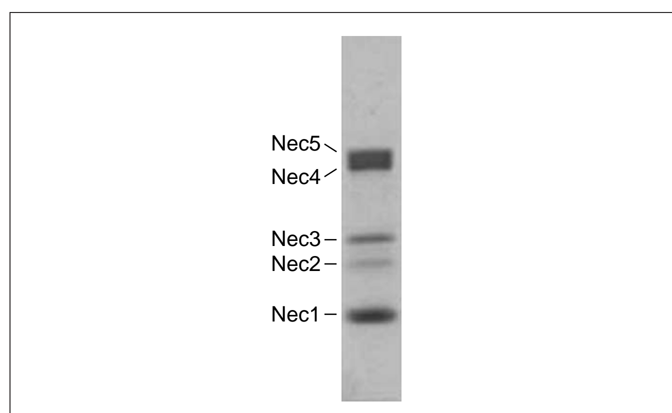

Figure 1. Electrophoresis of nectar proteins of ornamental tobacco on a 12% Laemmli gel.

analysis shows that it has a subunit molecular mass of 29 kDa. To understand the biochemistry of NEC1, we purified the protein from nectar and antiserum was raised against this protein. Immunoblot analyses showed that NEC1 was not related to the other nectar proteins and that the expression of *NEC1* was restricted to the nectary and, at a much lower level, the ovary [15]. No NEC1 was found in leaves, stems or roots or in any other floral organ. The expression of Nectarin I was also found to be developmentally regulated. It is expressed in nectary tissues, but only while nectar is being actively secreted from the nectary [25].

N-terminal sequence analysis showed that NEC1 is related to wheat germin, a protein that was first identified in germinating wheat seedlings [26]. In more recent studies, germin-like proteins (GLPs) were found to be widely distributed in all plants that have been examined. The function of germins and GLPs has received much attention [27–29], and recent theories have centered on the role of these proteins as defense factors [30–32].

To understand the functions of Nectarin I, we studied the *Arabidopsis* germin gene family. Surprisingly, we found that *Arabidopsis* has 32 genes that encode germin-like proteins [33–35]. Analysis of several of these *Arabidopsis* GLP genes in transgenic plants indicates that they are differentially expressed (C. Carter and R.W. Thornburg, unpublished). Following purification of NEC1 to homogeneity, the protein was characterized as a unique, manganese superoxide dismutase [25]. The enzymatic activity of superoxide dismutase removes superoxide and while generating hydrogen peroxide (Eqn 1, Box 1).

$$2O_2\cdot + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{[Eqn 1]}$$

Table 2. Genes described in this review

Gene sequence	Source	GenBank Accession	Refs
NEC1 cDNA	L × S8[a]	AF411917	[38]
NEC1 gene	*Nicotiana plumbaginifolia*	AF132671	[15]
NEC3 cDNA	L × S8[a]	AF492468	[43]
NEC5 cDNA	L × S8[a]	AF503441	[42]
NEC5 gene	L × S8[a]	AF503442	[42]

[a]L × S8 = ornamental tobacco interspecific cross *Nicotiana langsdorfii* × *Nicotiana sanderae* line 8.

Box 1. Source of superoxide in the nectary

We have investigated possible sources of superoxide in nectary tissues and have identified an enzymatic activity that produces high levels of superoxide (C. Carter and R.W. Thornburg, unpublished). The production of superoxide is localized to and is constitutively expressed in nectaries of mature flowers. This enzymatic activity requires NADPH as a substrate and NADH is not used. Enzymatic activity is not inhibited by either sodium azide or sodium cyanide but is inhibited by diphenylene iodonium (DPI), indicating that the superoxide-generating activity probably results from a classical NADPH oxidase [51–53]. Recently, we have isolated a 601 nt cDNA that we have termed *NOX1*. This fragment encodes a portion of a $gp91^{phox}$ from mature nectary mRNA [54–56]. Further analysis is in progress.

Because superoxide dismutases can generate hydrogen peroxide, we examined nectar for the presence of this compound. This analysis demonstrated that hydrogen peroxide accumulates to high levels, up to 4 mM, in the nectar of ornamental tobacco [25]. This is 40 times the level produced by human neutrophils in response to microbial attack [36]. These levels are also toxic to microorganisms (C. Carter and R.W. Thornburg, unpublished) and suggest that nectar might play a defensive role in protecting the gynoecium and developing ovules from microbial attack [37]. The hydrogen peroxide levels might also keep nectar palatable for visiting pollinators by limiting microbial growth, thereby preventing toxin buildup and reducing the breakdown of sugars and amino acids by microbial metabolism. Western blot analysis of nectars from ~20 other plant species revealed putative NEC1 homologs in nearly half of these species [25], indicating that the protective role of NEC1 might be wide spread throughout the plant kingdom.

A cDNA and gene encoding NEC1 were isolated (Table 2) and the *NEC1* promoter was analyzed in transgenic plants [38]. These studies demonstrated that the nectary has 15- to 100-fold higher levels of marker gene expression than any other plant organ. Therefore, the *NEC1* gene is strongly and uniquely expressed in nectary tissue. Developmentally, the expression of the *NEC1* promoter precedes nectar secretion by 12 to 24 h and is most active at times when nectar is being actively secreted from the nectary tissues. These results were also confirmed by reverse transcriptase–PCR (RT–PCR) and are summarized in Table 3.

Analysis of a series of promoter deletions revealed that nectary-specific expression is a result of multiple promoter elements. One of these elements is responsible for activating gene expression and contains a consensus MYB binding site [38]. We have recently isolated several nectary-expressed cDNAs that encode a MYB305-like protein. MYB305-like proteins are known to regulate the expression of a variety of floral genes including flavonoid biosynthetic genes in several species [39–41]. The promoters of several other nectary-expressed genes also contain MYB binding sites [38,42].

Nectarins II and III

We used similar methods to define the functions of other nectar proteins. N-terminal sequencing of the Nectarin II

Table 3. Spatial and temporal expression of nectary genes[a]

	Plant tissues[b]										Nectary stage[c]					
Gene	L	R	S	Pe	Pi	St	An	Se	O	N	2	6	8	10	12	PF
NEC1	–	–	–	–	–	–	–	–	+	+++	–	–	±	+	+++	–
NEC3	–	–	–	–	–	++	±	–	++	++	+	++	++	++	++	–
NEC5	–	–	–	–	–	–	–	–	–	+++	–	–	+	+++	++	–

[a] – indicates no expression; ±, +, ++, +++ indicates ambiguous, low, medium and high levels of expression, respectively.
[b]Plant tissues: L, leaf; R, root; S, stem; Pe, pedicel; Pi, pistil; St, stigma/style; An, anther/filament; Se, sepals; O, ovary; N, nectary.
[c]Nectary stage: 2, Stage 2 (early floral bud); 6, Stage 6 (immature nectary, filling stage); 8, Stage 8 (immature nectary, mid-maturation stage); 10, Stage 10 (immature nectary, early maturation stage, pre-secretory); 12, Stage 12 (mature nectary, secretory stage); PF, nectaries from post-fertilized flowers.

(NEC2; 35 kDa) and the Nectarin III (NEC3; 40 kDa) proteins revealed that they both share identity with dioscorin, the major soluble protein of yam tubers [43]. The NEC3 N-terminal sequence closely matched the N-terminus of dioscorin and the NEC2 N-terminal sequence matched a region of dioscorin that was 48 amino acids downstream of the mature NEC3 N-terminus, indicating that NEC2 is a degradation product of NEC3 [43]. After isolation of a cDNA encoding NEC3, complete sequence analysis confirmed the identity of NEC3 as a dioscorin-like protein. Further, MALDI-TOF mass fingerprinting of tryptic peptides derived from the purified NEC3 protein confirmed that the *NEC3* cDNA encoded the observed NEC3 protein.

In yam roots, dioscorin has been associated with several functions including carbonic anhydrase, monodehydroascorbate reductase, trypsin inhibitor, and direct antioxidant activities [44–47]. Therefore we tested NEC3 for these same enzymatic activities. Using in-gel staining methods, we demonstrated that the NEC3 protein is bifunctional and has carbonic anhydrase and monodehydroascorbate reductase activities. Further, there is strong identity between NEC3 and human carbonic anhydrase [48] and all the active site residues from the human enzyme are conserved in NEC3. This suggests that NEC3 and human carbonic anhydrase might share a common mechanism [43,48].

To determine temporal and spatial expression patterns of the *NEC3* transcripts, all major floral and vegetative organs were examined using RT–PCR. As summarized in Table 3, *NEC3* is expressed most strongly in the nectary gland; however, *NEC3* is also expressed at lower levels in the ovary, stigma, style, floral tube, and at very low levels in the anthers and filaments. The *NEC3* transcript was not expressed in other parts of the plant. Temporal analysis shows that the *NEC3* transcripts are expressed at low levels in the nectaries at the early flower bud stage and in increasing amounts throughout subsequent stages of nectary development. Following fertilization, *NEC3* transcript expression is abolished. This mode of expression differs from that of *NEC1* and suggests that different mechanisms control *NEC1* and *NEC3* expression.

The physiological roles of NEC3 in nectar can be inferred from its biochemical activities [43]. The carbonic anhydrase activity stabilizes the pH of nectar using the same buffer system that animals use in their blood. Because the superoxide dismutase (SOD) enzymatic activity consumes protons (Eqn 1), there is a propensity of nectar to become basic. Thus, carbonic anhydrase activity provides a pH-balanced meal for visiting pollinators and also maintains physiological conditions for proper function of nectar enzymes. The monodehydroascorbate reductase activity is intimately involved in the biochemistry of the nectar redox cycle (Box 2).

Nectarin V

The largest tobacco nectar protein is Nectarin V (NEC5). BLAST analysis of the NEC5 N-terminal sequence failed to identify any potential homologs. Therefore, degenerate oligonucleotides were designed based upon the N-terminal sequence; these permitted us to isolate partial genomic and near full-length cDNA sequences [42]. Tryptic peptide mass fingerprint analysis of the NEC5 protein confirmed that the *NEC5* genomic and cDNA sequences encode the observed protein.

BLAST searches of the complete translated NEC5 protein sequence identified it as a berberine bridge enzyme (BBE)-like protein. Berberine bridge enzyme (BBE; reticuline oxidase) catalyzes the first step in the biosynthesis of benzophenanthridine alkaloids in plants [49,50]. However, alkaloids are not present in the nectar of ornamental tobacco [42] suggesting that NEC5 might not be involved in alkaloid biosynthesis in nectar. One additional feature of the BBEs is that they contain a conserved, covalently bound FAD moiety that is required for activity. This sequence is also conserved in NEC5 and spectroscopic analysis confirmed that NEC5 contains a covalently bound flavin. In addition to identifying BBE, BLAST searches of the *NEC5* sequence also identified two closely related proteins that were annotated simply as carbohydrate oxidases (GenBank Accession number AF472608 and GenBank Accession number AF472609). Therefore, we evaluated the NEC5 protein for carbohydrate oxidase activity. This analysis demonstrated that NEC5 contains glucose oxidase activity. Although *NEC5* showed activity with glucose, neither galactose nor mannose could serve as substrates to generate hydrogen peroxide [42]. This glucose oxidase activity oxidizes glucose and generates additional hydrogen peroxide that probably contributes toward the antimicrobial levels of hydrogen peroxide found in nectar. We have preliminary evidence that NEC5 can also use dehydroascorbate as a terminal electron acceptor instead of oxygen, resulting in the regeneration of ascorbate from dehydroascorbate (C. Carter and R.W. Thornburg, unpublished).

Both the spatial and temporal expression profiles of *NEC5* closely mirror those of *NEC1* (summarized in Table 3). Because this pattern of expression is nearly identical with that of *NEC1*, we searched for and verified that the *NEC5* promoter also contains a *MYB*-binding site

Box 2. Nectar redox cycle

Nectar contains high levels of hydrogen peroxide (up to 4 mM) that are maintained by the action of NEC1 and NEC5. However, hydrogen peroxide is notoriously unstable in the presence of metal ions and breaks down to form hydroxyl free radicals [OH·] (Figure I) [57,58]. These hydroxyl free radicals are extremely deleterious in biological systems attacking membranes, proteins and DNA. Fortunately, nectar also contains the antioxidant, ascorbate, which scavenges these free radicals by reducing them to the non-toxic hydroxide anions [OH^-]. This results in the production of monodehydroascorbate (MDHA) [59,60]. Because ascorbate is present in tobacco nectar at a significantly lower concentration than hydrogen peroxide, we hypothesize that there must be a mechanism (i.e. NEC3) to regenerate ascorbate levels in nectar thus avoiding a significant decline in levels of this antioxidant that would result in the buildup of additional toxic hydroxyl free radicals.

There is also a well-understood non-enzymatic disproportionation reaction of MDHA that produces one molecule of ascorbate (reduced) and one molecule of the dehydroascorbate (DHA; oxidized). However, this non-enzymatic reaction is kinetically slow and is unlikely to provide a sufficient return of monodehydroascorbate to ascorbate to avoid the eventual buildup of toxic hydroxyl free radicals. Therefore, the monodehydroascorbate reductase activity of NEC3 is likely to maintain ascorbate concentrations in nectar by converting monodehydroascorbate directly back into ascorbate.

Nectarin V is a glucose oxidase that also generates high levels of hydrogen peroxide using oxygen as a terminal electron acceptor. Preliminary studies (C. Carter and R.W. Thornburg, unpublished) indicate that this protein can also use dehydroascorbate as a terminal electron acceptor, thereby converting any DHA back into ascorbate to complete the ascorbate cycle (broken arrows in Figure I).

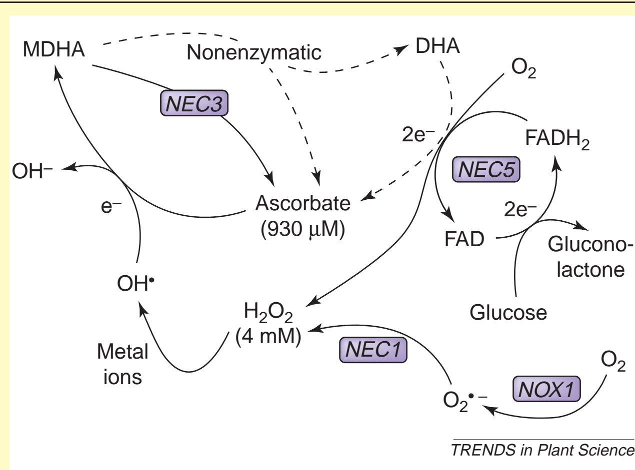

Figure I. Nectar redox cycle.

that is similar to sites present in the *nec1*gene and the promoters of other nectary-expressed genes [42].

Summary

Insect pollination has been widely established as among the most important aspects of fruit and seed production. It is well known that increasing insect visitation results in increased pollination efficiency and subsequently in increased seed set (yield). Any attempt to manipulate insect visitation to affect yield must have as its first step a complete understanding of the components that plants use to attract insects. We have focused on the biochemistry of nectar. Our studies indicate that there is a limited array of proteins that accumulate in nectar of ornamental tobacco, yet these proteins generate a novel biochemistry that we propose serves primarily to protect the gynoecium from infection by microorganisms. The NEC1 protein appears to be common among angiosperms and it, together with NEC5, generates high levels of hydrogen peroxide in nectar. We hypothesize the high level of hydrogen peroxide maintains the rich nectar in an axenic state either by inhibiting the growth of microorganisms or by directly killing them.

High levels of hydrogen peroxide in the presence of metal ions produce deleterious free radicals (hydroxyl radical, OH·). In a novel oxidation–reduction cycle, which we have termed the 'nectar redox cycle', ascorbate, NEC3 and possibly NEC5 detoxify these free radicals. In addition, NEC3 maintains the pH of nectar by using the same buffering system found in animal sera. By understanding the biochemistry of nectar, we are not only gaining new insights into novel mechanisms of floral defense and the co-evolution of plants and animals, but we are also beginning to gain inroads into the manipulation of nectar biochemistry. We eventually hope to use these insights to affect pollinator visitation and ultimately to increase yield in insect-pollinated angiosperms.

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