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REVIEW

Biochemistry of Nectar Proteins

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Floral nectar is a rich concoction of sugars, amino acids, vitamins, lipids, and other ingredients that is usually secreted at the base of a flower. Nectar is offered freely to attract large numbers of visiting pollinators to the flower. These visiting pollinators then transfer pollen between flowers, completing the plant's sexual cycle to produce seeds. However, in addition to transferring pollen, visiting pollinators also transfer microorganisms between flowers when they drink nectar. Because nectar is so rich in metabolites and is secreted at the flower's base surrounding the ovary, nectar offers a potential infection site that could severely reduce the plants' fecundity if microorganisms invade. To circumvent this problem, plants have developed strategies to deal with microbial invaders. In ornamental tobacco, a small array of proteins is secreted into floral nectar. We have isolated and characterized each of these proteins and have identified a novel biochemical pathway, the "nectar redox cycle", in ornamental tobacco floral nectar that functions to reduce the growth of microorganisms in the metabolically rich floral nectar. The identification of proteins in nectar has ushered a new area of plant biology that functions to maintain floral integrity.

Reproductive seed set is the culmination of the plant's life cycle resulting in genetically new individuals capable of reestablishing species in a new locale and at a later time. For many angiosperms, this process is mediated by animal vectors that transfer pollen among flowers, thereby increasing plant fecundity. Animal pollinators (primarily insects, but also birds and mammals) are attracted to the flowers of many angiosperms by the reward of nutritionally rich floral nectar that is secreted into the floral base by a remarkable floral gland, the nectary.

Floral nectar is a rich source of sugars, amino acids, vitamins, organic acids, metal ions, and other metabolic components [30] and serves as a reward for pollinator visitation. However, because flower visitors are not sterile, they not only transfer pollen during these visits but they can also transfer any microorganisms that they carry. Despite the fact that flowers remain open, offering metabolically rich fluids to pollinators over an extended timeframe, infections of the floral gynoecium are relatively rare in plants. This implies that there must be an active defense system in nectar to reduce such infections [40].

To examine this possibility in greater detail, we began studies to determine whether there were proteins that accumulated in the nectar of plants. Using ornamental tobacco as a model system, we verified that proteins did indeed accumulate in the floral nectar [7]. These proteins, termed nectarins, accumulate in ornamental tobacco nectar to concentrations of 250 mg/mL of nectar and were named Nectarin I through Nectarin V in order of increasing molecular mass. Following N-terminal sequencing and proteomic analysis, the individual nectarins were identified, characterized, and cloned and their biochemistry unraveled.

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Nectarin I

Nectarin I (*NEC1*), a 29-kDa monomer, is the most abundant nectar protein in ornamental tobacco. From its N-terminal amino acid sequence, it was identified as a novel germin-like protein [7]. To obtain a clearer picture of the tobacco Nectarin I gene family, we examined the *Arabidopsis* genome for related genes. As it turns out, *Arabidopsis* contains a large family of germin-like proteins (GLPs) with at least 27 different family members [6, 9]. In addition, germin-like proteins have been identified in all plants from mosses through gymnosperms and grasses [29, 17, 41]. While some GLPs, including the original wheat germin, have oxalate oxidase activity [28], Nectarin I does not. Instead, Nectarin I was found to have superoxide dismutase activity [10]. Because most superoxide dismutases are metal-binding proteins [24, 42, 39], we analyzed purified Nectarin I for metals. This analysis demonstrated the presence of manganese in the Nectarin I protein. We were also able to remove all metals from the Nectarin I protein and reconstitute enzyme activity only when supplemented with manganese and not with iron or with copper or zinc or both copper and zinc together.

To evaluate its distribution in the plant kingdom, we examined floral nectar from 15 species representing 11 plant families that were present in local greenhouses, suggesting that Nectarin I may be found broadly throughout the plant kingdom. Nectarin I was also found to be highly stable. It retained full activity when heated at 90°C for 15 min. This thermostability was exploited as a novel method of purification of Nectarin I. Further, in Western blots, without boiling, this protein was completely stable in the presence of sodium dodecyl sulfate (SDS) and migrates as an apparent 165-kDa oligomeric protein. Wheat germin, which shares >60% amino acid identity with Nectarin I, is a hexomeric protein [19], and other closely related GLPs are probably also hexomeric proteins with molecular masses approximately 170 kDa.

Recently, we identified the source of superoxide production in the nectary as a developmentally regulated NADPH oxidase [8]. Those studies also demonstrated the direct antimicrobial action of H_2O_2 in nectar and confirmed the protective function of plant nectar against microbial attack [40].

The expression of the nectarins is also tightly regulated. In the case of Nectarin I, the protein is expressed uniquely in the nectary gland and only at times when nectar is actively being secreted from the nectary gland [7, 11]. To confirm expression of the Nectarin I gene, the *NEC1* promoter was isolated and reporter constructs prepared. These constructs were moved into transgenic plants, and expression studies demonstrated that the *NEC1* gene is expressed exclusively in actively secreting nectary tissue [11]. These studies also demonstrated that multiple promoter elements were present in the promoter. One of these elements was responsible for the temporal pattern of expression and contained a consensus MYB-binding site. The other element was responsible for limiting expression of the gene in petals.

Recently, we have identified a transcription factor that we term MYB305. *MYB305* is highly expressed in mature nectaries, but is not expressed in nectaries from immature flowers. This transcription factor is nuclear localized, supports transcription in yeast, and binds to the consensus MYB-binding site within the *NEC1* promoter (Liu and Thornburg, manuscript in preparation). Further, removing just the MYB-binding site from the *NEC1* promoter renders the promoter non-functional. Thus, it appears that Nectarin I is regulated by this nectary-expressed transcription factor.

Nectarin II and Nectarin III

Nectarin II (*NEC2*; 35 kDa) and Nectarin III (*NEC3*; 40 kDa) are moderately sized proteins present in the nectar of ornamental tobacco plants. N-terminal sequencing of both of these proteins revealed that each of these proteins share identity with the same protein, dioscorin, which is the major soluble protein of yam tubers [12]. This suggests that the smaller Nectarin II may be a breakdown product of Nectarin III. It also suggests a novel method for cloning of these nectar proteins.

We prepared degenerate oligonucleotides that matched the N-terminal sequences of both Nectarin II and Nectarin III. Using reverse transcription polymerase chain reaction (RT-PCR) of nectary mRNA, we were able to generate a 185-nt cDNA fragment that had identity to both the Nectarin II and Nectarin III N termini. This fragment was used as a probe to screen a nectary cDNA library, and a full-length cDNA encoding both nectarins was obtained [12]. Analysis of this cDNA revealed that the cDNA encoded a protein of 274 amino acids with a 25-amino acid N-terminal signal sequence. The Nectarin II N terminus matches the sequence of the translated protein beginning with amino acid 74. Thus, Nectarin II is cleaved at Glu^{73} and is missing the N-terminal portion. The protease responsible for this cleavage is unknown, as is the subcellular location where the cleavage occurs.

To confirm that the isolated cDNA did indeed encode the Nectarin III protein, we performed matrix-assisted laser desorption /ionization–time of flight mass spectrometry analysis of the Nectarin III protein. From this analysis, we identified nine peptides that matched the predicted masses from the translated sequence of the Nectarin III clone. Together with the N-terminal sequences, these peptides covered >40% of the translated amino acid sequence of the

clone, and we concluded that the isolated clone did indeed encode the Nectarin III protein.

Blast analysis of the Nectarin III sequence revealed a set of proteins with high identity to Nectarin III. The most closely related sequences were unidentified proteins from *Arabidopsis*, but another group of highly related sequences were the root storage protein genes (dioscorins) isolated from *Dioscorea alata* and *Dioscorea cayenensis* [12]. Dioscorins have been associated with several biochemical functions, including carbonic anhydrase, monodehydroascorbate reductase, trypsin inhibitor, and direct antioxidant activities [14, 21, 22, 23].

We next determined whether the Nectarin III protein had any of these biochemical activities. Using native gel electrophoresis coupled with in-gel enzyme assays, we were able to determine that Nectarin III had both carbonic anhydrase and monodehydroascorbate reductase activity. Further, when we isolated the bands from the native gels and reran those bands on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels, we observed that the Nectarin III and Nectarin II bands were the only proteins present in activity bands excised from the native gels. In addition, we also tested whether Nectarin III was a trypsin inhibitor; however, we were unable to find any evidence of trypsin inhibitor activity.

Based upon these enzymatic activities, we can infer the physiological role of Nectarin III in floral nectar. The carbonic anhydrase activity of *NEC3* may function to buffer the pH of nectar. Because nectar has a propensity to become basic due to the detoxification of hydroxyl free radicals, the stabilization of nectar pH would insure that the biochemical properties of nectar proteins function efficiently. In addition, buffering nectar to a physiological pH would also provide a pH-balanced meal for visiting pollinators. It is interesting to note that the plant nectary uses exactly the same mechanism (carbonic anhydrase) to buffer the pH of nectar that animals use to buffer the pH of blood.

To evaluate the temporal and spatial expression of Nectarin III, we utilized RT-PCR. In contrast to Nectarin I, which is expressed uniquely in the nectary gland and only at times when nectar is actively flowing, Nectarin III is expressed in many floral organs including the stigma/style, anther/filament, floral tube and ovary, and in the nectary; it is expressed throughout nectary development, from early times until anthesis. However, Nectarin III expression is dramatically turned off after fertilization. Because of the differences in expression between Nectarin I and Nectarin III, we conclude that there must be multiple molecular mechanisms for expression of genes in the nectary gland.

Nectarin IV

Nectarin IV and Nectarin V are of nearly similar molecular mass and run in close proximity on SDS polyacrylamide gels. N-terminal sequencing of the Nectarin IV protein was not successful, suggesting that this protein has a blocked N terminus. To generate sequence information that might permit cloning and analysis of the Nectarin IV protein, we subjected the Nectarin IV protein to tandem mass spectrometry sequencing. This analysis identified one 14-amino acid peptide that matched the identity of a tomato xyloglucan-specific endoglucanase inhibitor protein (XEGIP). Two additional peptides were also identified that matched the sequence of a protein that is the potato homolog of the tomato XEGIP.

Using this information, we designed a strategy to clone the tobacco nectary XEGIP. We identified all known homologs of the tomato XEGIP that were present in the various databases and aligned them using a ClustalW algorithm. This permitted us to identify conserved nucleotide sequences and to design a pair of oligonucleotide primers for PCR. The resulting PCR product was cloned and sequenced. This permitted us to design additional oligonucleotides specific for the Nectarin IV sequence. These oligonucleotides, together with library vector sequences, permitted the isolation of full-length 5′ and 3′ ends.

Examination of the N terminus of Nectarin IV revealed a predicted 22-amino acid signal peptide responsible for secretion of the protein. The first amino acid following the predicted signal peptide cleavage site is Gln-23. N-terminal glutamine residues are frequently converted into pyroglutamate residues by glutamine cyclase enzymes [31, 38] resulting in a blocked N terminus, which is consistent with our earlier observations from N-terminal analysis.

To determine whether the Nectarin IV protein had xyloendoglucanase (XEG) inhibitory activity, the Nectarin IV protein was purified and incubated with the xyloglucan-specific endoglucanase (XEG) from *Aspergillus aculeatus*. These assays demonstrated that Nectarin IV was a potent inhibitor of XEG, confirming its activity as an XEGIP. Further, Nectarin IV was very tightly bound by the XEG with a Ki of 0.35 nM. Because many fungi express hemicellulose-degrading enzymes such as the XEG in an attempt to colonize and infect plant tissues, we hypothesized that the Nectarin IV XEGIP activity likely functions to inhibit fungal endoglucanases from degrading the hemicelluloses in the nectary cell walls. We envision this process to be analogous to the inhibition of pathogenic fungal polygalacturonases by plant polygalacturonase-inhibiting proteins [34].

To evaluate its temporal and spatial expression, the levels of Nectarin IV mRNA were examined in nectaries using RT-PCR. Comparisons were made to glyceraldehyde-3-phosphate dehydrogenase mRNA, which is invariant in the ornamental tobacco nectary throughout its development and is also invariant in other floral tissues. These studies showed that the Nectarin IV mRNA is exclusively expressed in the nectary tissues. No other floral organ, nor

roots, stems, or leaves showed expression of the Nectarin IV mRNA. The temporal expression of Nectarin IV was also different from either Nectarin I or Nectarin III. Unlike Nectarin III, Nectarin IV showed no expression at early times of nectary development, and unlike Nectarin I, the expression of Nectarin IV did not decline after fertilization. Instead, the Nectarin IV mRNA began to be expressed about floral stage S9 and showed increasing expression throughout floral development and even showed an increased expression after pollination. This suggests that inhibition of fungal hemicellulases may be even more important after fertilization than it is at anthesis.

Nectarin V

On SDS-PAGE gels, Nectarin V runs together with Nectarin IV as a broad doublet of approximately 65 kDa. To identify the Nectarin V protein, we separated nectar proteins on a high-resolution gel run for 12 h on an 18-cm 8% analytical SDS polyacrylamide gel. This analysis resolved the Nectarin IV/V doublet into five individual protein bands [13]. Because all of the nectarins are glycoproteins, this variation in molecular mass is thought to be due to incomplete glycosylation of the various proteins. Subsequently, each of these bands was then excised from the gel and subjected to N-terminal sequence analysis. The two lowest molecular mass bands failed to provide an N-terminal sequence that we interpreted as being Nectarin IV isoforms with blocked N termini. The three upper bands revealed an identical N-terminal sequence, and these bands were named Nectarin Va, Vb, and Vc in terms of increasing molecular mass.

To clone the Nectarin V protein, we designed a pair of degenerate oligonucleotides that matched the ends of this 22-amino acid N-terminal sequence. These oligonucleotides were used in an RT-PCR reaction to amplify first-strand, nectary-expressed cDNAs. After amplification, a small fragment was identified and this fragment was blunt-end-cloned into a plasmid vector. After sequencing of this fragment, we determined that the 65-nucleotide fragment perfectly matched the first 22 amino acids of the mature protein. After several unsuccessful attempts to screen a nectary cDNA library and a *Nicotiana plumbaginifolia* genomic library, we attempted to increase the size of our cloned fragment using inverse PCR (I-PCR). Therefore, a pair of outwardly rectifying oligonucleotides that matched the 65-nucleotide internal Nectarin V fragment was designed. Ornamental tobacco genomic DNA was isolated, digested with *Sau*3A I, and self-ligated using T4 DNA ligase. The resulting circular fragments were used as PCR templates with the second oligonucleotide pair. This process resulted in a 538-nucleotide fragment that was cloned and sequenced. This fragment overlapped with the first, confirming that the sequences were related. A second round of I-PCR using another set of outwardly rectifying oligonucleotides was carried out using genomic DNA digested with *Taq*I followed by self-ligation. This second round of I-PCR resulted in a 1.6-kB fragment that was also cloned and sequenced. This fragment contained about one half of the Nectarin V coding sequence and 787 nucleotides of flanking sequences upstream of the ATG start codon. This larger Nectarin V fragment was used to screen a nectary cDNA library and a single plaque was identified, purified, and sequenced. This cDNA lacked approximately 360 nucleotides from the 5′ end, including the first 305 amino acids. However, alignments with the I-PCR fragments revealed the full-length open reading frame of the Nectarin V protein.

To confirm that the cloned Nectarin V gene encoded the nectary-expressed Nectarin V protein, we performed tryptic peptide fingerprint mass spectrometry on each of the three Nectarin V isoforms. From this analysis, we identified 22 peptides that matched the masses predicted from the translated sequence of the Nectarin V gene. Together, these peptides covered 39.4% of the total amino acid sequence of the mature Nectarin V protein, and we concluded that the cloned Nectarin V gene did indeed encode the Nectarin V protein isolated from nectar.

BLAST analyses of the full-length translated Nectarin V protein sequence identified it as a member of the berberine bridge enzyme family (BBE-like). BBE ([S]-reticuine: oxygen oxidoreductase) was originally identified in California poppy [16] as an enzyme that catalyzed a major branch point in benzophenanthridine alkaloid biosynthesis in plants. Our BLAST analysis revealed that there are a large number of these proteins in the plant kingdom. *Arabidopsis* alone has 27 potential homologs; however, phylogenetic analysis reveals that the true BBEs form a separate clade from all of the *Arabidopsis* clones, suggesting that these *Arabidopsis* homologs may not have BBE activity. We also examined nectar for the presence of alkaloids and failed to find them. From these analyses, we concluded that Nectarin V is not likely to be involved in alkaloid biosynthesis in nectar.

Analysis of BBEs from poppies revealed the presence of a conserved, covalently bound FAD moiety that is required for activity. Nectarin V shows 58% identity with BBE in the vicinity of the FAD binding site, including the histidine that is modified by FAD [13]. To determine whether Nectarin V contains flavin, we purified Nectarin V from nectar and recorded the protein's fluorescence spectra. Both the excitation and emission maxima matched that of the poppy BBE [16]. To determine whether the flavin was covalently attached, nectar proteins were subjected to SDS-PAGE, and after electrophoresis; the proteins were examined for fluorescence. Only the 65-kDa Nectarin V protein

showed fluorescence, confirming the covalent attachment of the flavin to the Nectarin V protein.

In addition to characterizing Nectarin V as a BBE, the BLAST analysis also identified two clones that were that were distantly related to Nectarin V. These proteins were annotated simply as carbohydrate oxidases from lettuce (*Lactuca sativa*) and from sunflower (*Helianthus annuus*). To determine whether Nectarin V might be a carbohydrate oxidase, we ran nectar proteins on native gels and incubated them in glucose oxidase substrate solution in the presence of various sugars. In the presence of glucose, we observed an intensely staining band. When this band was excised from the gel and run on an SDS-PAGE gel, a single protein band was observed that co-migrated with the Nectarin V protein. In the presence of other sugars, galactose and mannose, no staining was observed. Thus, we concluded that Nectarin V was a glucose oxidase that used oxygen as a terminal electron acceptor to generate hydrogen peroxide. We further found that in the absence of oxygen, the Nectarin V protein could use dehydroascorbate as a terminal electron acceptor converting dehydroascorbate back into ascorbate.

To evaluate the temporal and spatial expression patterns of Nectarin V, we utilized RT-PCR. The pattern of expression was nearly identical with that of Nectarin I [7]. Because we had earlier predicted that a MYB transcription factor was responsible for expression of Nectarin I [11], we examined the promoter of Nectarin V for MYB binding sites to determine whether both genes may be coordinately regulated. This search revealed the presence of a consensus MYB-binding site within the Nectarin V promoter, and its presence raises the intriguing possibility that both Nectarins I and V are expressed by the same MYB transcription factor.

Nectar Redox Cycle

The nectar of ornamental tobacco, like that of most plants, is a very rich mixture of sugars, amino acids, vitamins, organic acids, metal ions, and other compounds that are required for normal growth of biological organisms. Because this rich fluid is freely offered to visiting pollinators, it is not surprising that pollinators take advantage of these offerings to feed themselves and their nests. However, for the plant, this is a double-edged sword, for the visiting pollinators are not sterile and the nectar is offered to draw these pollinators to the plant's reproductive tract.

In spite of this enriched media surrounding the floral gynoecium and the fact that insects are not sterile, it is a general observation that infections of flowers are relatively rare. This simple observation has tremendous implications. It suggests that plants have developed potent defense systems to protect nectar and the gynoecium that the nectar bathes from infection. The proteins that accumulate in ornamental tobacco nectar represent one such system, termed the 'Carter–Thornburg' nectar redox cycle (Fig. 1). The nectar redox cycle is a novel biochemical pathway present in soluble nectar that functions to generate very high levels of hydrogen peroxide which is antimicrobial and also permits the plant to deal with these very high levels of reactive oxygen species.

The nectar redox cycle is initiated by a membrane-bound NADPH oxidase that converts molecular oxygen into superoxide [8]. This NADPH oxidase is developmentally

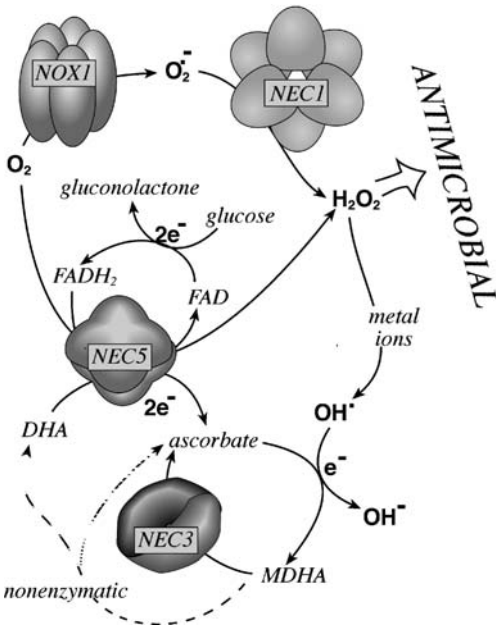

Fig. 1 'Carter–Thornburg' nectar redox cycle. The nectar redox cycle is initiated by a membrane bound NADPH oxidase (*NOX1*) that converts molecular oxygen into superoxide. Nectarin I (*NEC1*) functions as a superoxide dismutase that disproportionate superoxide into hydrogen peroxide, resulting in the accumulation of hydrogen peroxide in nectar. Simultaneously, Nectarin V (*NEC5*) functions as a glucose oxidase that utilizes a covalently bound FAD moiety and glucose in a two-electron reduction of molecular oxygen to generate additional hydrogen peroxide. From these two reactions, the resulting hydrogen peroxide accumulates in soluble nectar to very high levels, up to 4 mM. Nectarin III (*NEC3*) is a monodehydroascorbate reductase that is capable of directly converting MDHA into ascorbate. In addition, there is a non-enzymatic reaction that will also convert MDHA into ascorbate. If two molecules of MDHA interact in solution, the extra electron in one MDHA molecule can be passed to the second MDHA molecule. This results in the reduction of the first MDHA to ascorbate and the oxidation of the second MDHA to the two-electron-deficient dehydroascorbate (*DHA*). The enzyme Nectarin V can also utilize DHA as a terminal electron acceptor and in doing so converts the DHA back into ascorbate. Thus, the nectar redox cycle results in very high levels of hydrogen peroxide in soluble nectar and functions to reduce the growth of microorganisms in the metabolically rich floral nectar

regulated in the nectary and is expressed just prior to floral maturity. Subsequently, Nectarin I functions as a superoxide dismutase that disproportionates superoxide into hydrogen peroxide, resulting in the accumulation of hydrogen peroxide in nectar. Simultaneously, Nectarin V functions as a glucose oxidase that utilizes a covalently bound FAD moiety and glucose in a two-electron reduction of molecular oxygen to generate additional hydrogen peroxide. From these two reactions, the resulting hydrogen peroxide accumulates in soluble nectar to very high levels, up to 4 mM [10]! This level is 40 times the level of hydrogen peroxide produced by human neutrophils in response to microbial attack [36]. Because of its role as an antimicrobial factor, we have previously proposed that the production of hydrogen peroxide in soluble nectar serves to maintain floral nectar in an axenic or microbe-free state, thereby protecting the gynoecium, ovules, and developing seeds from pollinator-borne and airborne pathogens [40, 12].

There is a problem with hydrogen peroxide in biological systems. In the presence of metal ions, notably iron and copper, hydrogen peroxide is unstable and undergoes a well-characterized Fenton chemistry that results in the production of hydroxyl free radicals. Free radicals are notoriously bad in biological systems. They react indiscriminately with all types of biological molecules; they not only cross-link these molecules but also have other toxic effects on cells. Fortunately, the flower has developed a mechanism to detoxify any free radicals that are produced in nectar. The flower secretes the antioxidant ascorbate into nectar which can reacts with any free radicals that it encounters to abstract the extra electron from a hydroxyl free radical, thereby detoxifying the hydroxyl free radical into hydroxyl anions. In this process, ascorbate is reduced to monodehydroascorbate (MDHA).

The amount of ascorbate in nectar is 900 μM, while the amount of hydrogen peroxide is 4 mM, so if there were not some way to convert MDHA back into ascorbate, the plant would face the same free radical problem after the ascorbate was used up. There are several mechanisms that permit regeneration of ascorbate in nectar. Initially, the enzyme Nectarin III is a monodehydroascorbate reductase that is capable of directly converting MDHA into ascorbate. In addition, there is a non-enzymatic reaction that will also convert MDHA into ascorbate. If two molecules of MDHA interact in solution, the extra electron in one MDHA molecule can be passed to the second MDHA molecule. This results in the reduction of the first MDHA to ascorbate and the oxidation of the second MDHA to the two-electron-deficient dehydroascorbate (DHA). As we have previously demonstrated [13], the enzyme Nectarin V can also utilize DHA as a terminal electron acceptor and in doing so converts the DHA back into ascorbate. Thus, the nectar redox cycle results in very high levels of hydrogen peroxide in soluble nectar and permits the plant to deal with the toxic products of these high levels of hydrogen peroxide.

It is clear that these high levels of hydrogen peroxide are antimicrobial. Nectar inhibits the growth of bacteria, while nectar treated with catalase to destroy the hydrogen peroxide does not inhibit bacterial growth [40, 8]. With these high levels of hydrogen peroxide present in nectar, the food source for many insects, one must ask how pollinating insects are able to deal with these levels of hydrogen peroxide. It appears that insects are able to handle hydrogen peroxide because both peroxidase and catalase activities appear to be abundant in the gut and Malpighian tubules of insects [18, 4, 15, 1].

Other Solutions to the Nectar Infection Problem

The problem confronted by plants in opening their reproductive tract to visiting pollinators is indeed a serious problem. Floral infections can and do occur, although they are not highly common. Often, these infections occur through infection of the floral nectary. Because the floral nectary has openings for nectar efflux, it is possible that these nectary pores can provide a channel for infection to spread. Classical examples of floral infection through the nectary include the fire blight infection (*Erwinia amylovora*) in apples and pears [5] as well as *Aspergillus flavus* infection of cotton [26, 25].

Thus, plants need to express nectar-defense proteins to protect these susceptible organs from becoming infected. While the nectar redox cycle functions adequately to do this in ornamental tobacco, it is known that this biochemical pathway is not found in all plants. Using a Western blot assay, Carter and Thornburg [10] examined the nectar of 15 species from 11 plant families that were present in local greenhouses for the presence of Nectarin I. While immuno-cross-reactive proteins were identified in approximately 65% of these species, but not all species showed the presence of this protein. Thus, there must be other mechanisms provided by other defense proteins or other antimicrobial factors that mediate the floral defense of other species. Unfortunately, analysis of nectar proteins is still in its infancy. In only two plants have the full complement of nectar proteins been characterized—ornamental tobacco and in leek (*Allium porrum*).

Leek

In leek, Peumans et al. [33] identified two defense-related proteins that accumulate in leek nectar. A 13-kDa protein that was present at about 150 μg/mL was identified as a mannose-binding lectin. This protein is structurally and

evolutionarily conserved among the monocots. As with other lectins, it binds the mannose oligosaccharides of glycolipids, glycoproteins, and other cell-associated carbohydrates, agglutinating the cells that contain these moieties. Proteins from this family have previously been shown to have antibiotic properties and are especially active against nematodes and insects with piercing-sucking mouthparts [20, 35, 37]. The mannose-binding lectin was found to be expressed in the nectars of several *Allium* species (onion, shallot, leek) as well as the nectar of snowdrop (*Galanthus nivalus*); however, the nectars of orchids, lilies, and amaranths did not contain the mannose-binding lectin. When these investigators fed leek nectar to honeybees, they demonstrated that the nectar was not toxic to honeybees, although it was toxic to aphids and nematodes [33].

A second protein was also identified in leek nectar. Alliinase (alliin lyase) is the enzyme responsible for the production of the organo-sulfur compounds that cause the characteristic odor and flavor of allium plants [27]. This pyridoxal-phosphate-containing enzyme also produces antimicrobial sulfur-containing compounds [3, 32]. This enzyme converts a non-toxic substrate (alliin) into the toxic compound (allicin) that has activity against many different kinds of microorganisms including both Gram-positive and Gram-negative bacteria, fungi, parasites, and viruses [2].

Other Species

Many other species are known to contain proteins in their nectar. However, these proteins have generally not been characterized. The importance of avoiding infections in the vicinity of the gynoecium is so central to plant biology that there must surely be additional mechanisms of nectar defense that have not yet been identified. It is beginning to be an exciting time in nectar biochemistry that such mechanisms are coming to light.

Conclusion

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 that 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 free radicals). 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. The *NEC4* XEGIP functions to inhibit endoglucanases expressed by pathogenic fungi attempting to infect and colonize plant tissues. 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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