

Tobacco Nectarin III is a bifunctional enzyme with monodehydroascorbate reductase and carbonic anhydrase activities*

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Abstract

Tobacco plants secrete a limited array of proteins (nectarins) into their floral nectar. N-terminal sequencing of the Nectarin II (*NEC2*; 35 kD) and the Nectarin III (*NEC3*; 40 kD) proteins revealed that they both share identity with dioscorin, the major soluble protein of yam tubers. These sequences also revealed that *NEC2* is a breakdown product of *NEC3*. Using these N-terminal peptide sequences, degenerate oligonucleotides were designed that permitted the isolation of a partial *NEC3* cDNA. This cDNA was then used to probe a nectary specific cDNA library and a full-length *NEC3* cDNA clone was isolated. Complete sequence analysis confirmed the identity of *NEC3* as a dioscorin-like protein. MALDI-TOF mass spectrometric fingerprinting of tryptic peptides derived from the purified *NEC3* confirmed that this protein was encoded by the isolated cDNA. *NEC3* was shown to possess both carbonic anhydrase and monodehydroascorbate reductase activities. RT-PCR based expression analyses demonstrated that *NEC3* transcript is expressed throughout nectary development as well as in other floral organs. A proposed function in the maintenance of pH and oxidative balance in nectar is discussed.

Abbreviations: MDH, monodehydroascorbate; MDHR, monodehydroascorbate reductase; *NEC1*, Nectarin I; *NEC2*, Nectarin II; *NEC3*, Nectarin III; *NEC5*, Nectarin V

Introduction

Many angiosperms have developed a remarkable reproductive strategy that relies on attracting animals to transfer pollen from flower to flower. Animals are frequently attracted to flowers with rewards of nectar, a metabolically rich fluid produced by an often distinct, terminally differentiated floral organ termed the nectary gland. During its development, the nectary gland undergoes enormous restructuring of form and function, which culminates in the production of nectar at

floral maturity. The chemical composition of nectar varies considerably, but generally includes solutes such as sugars, amino acids, lipids, antioxidants, and metal ions (Baker and Baker, 1983). The presence of proteins in plant nectars has also been historically described (Beutler, 1935; Zimmerman, 1953; Frey-Wyssling *et al.*, 1954; Zauralov, 1969; Scogin, 1979); however, these reports have largely failed to identify or characterize these proteins. We have identified several proteins (nectarins) that are uniquely expressed in the nectar of ornamental tobacco and have begun to characterize these proteins (Figure 1).

Our previous work has characterized the most abundant of these nectar proteins, Nectarin I

*Dedicated to Professor John W. Baynes on the occasion of his 63rd birthday.

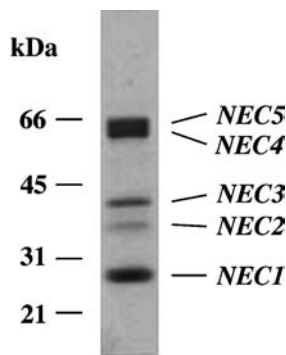


Figure 1. Ornamental tobacco nectar proteins displayed on an SDS PAGE.

(*NEC1*), as a novel germin-like protein (Carter *et al.*, 1999) that has superoxide dismutase (SOD) activity (Carter and Thornburg, 2000). We have also recently demonstrated that Nectarin V (*NEC5*) contains glucose oxidase activity (Carter and Thornburg, 2004). The SOD and glucose oxidase activities generate hydrogen peroxide at levels up to 4 mM (Carter and Thornburg, 2000). We have shown that these levels are antimicrobial (Carter *et al.*, in preparation), and we have hypothesized that the hydrogen peroxide produced by *NEC1* and *NEC5* functions as an antimicrobial defense to protect the gynoecium and developing ovules from microbial attack (Carter and Thornburg, 2000, 2004). The expression patterns of *NEC1* and *NEC5* are also unique. These genes begin to be expressed at late Stage 10 of nectary development approximately 12 h prior to anthesis (Carter and Thornburg, 2003, 2004). This is the stage when nectar just begins to flow from the nectary gland. *NEC1* and *NEC5* are strongly expressed when the flower opens (Stage 12) and continue to be expressed as long as the flower is secreting nectar. Nectar production ceases after floral pollination and *NEC1* and *NEC5* expression also ceases at this time. In addition, because the nectary gland also produces a rich variety of other antimicrobial proteins and peptides, we have proposed that a heretofore-unrecognized function of this floral organ is to protect the gynoecium and developing ovules from microbial attack (Thornburg *et al.*, 2003).

Until now, the identification of the other nectarins has remained elusive. Here we present N-terminal sequence data for *NEC2* and *NEC3* and an isolated cDNA that encodes both proteins. We

identify these proteins as a dioscorin-like protein and demonstrate enzymatic activities for *NEC3*. Finally, we propose a biochemical mechanism for the maintenance of pH and antioxidant status within nectar.

Materials and methods

Plant material

The ornamental tobacco plants used in this study were derived from an interspecific cross between *Nicotiana langsdorfii* 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) as well as *NEC1* (Carter *et al.*, 1999; Carter and Thornburg, 2000, 2003) and *NEC5* (Carter and Thornburg, 2004). Nectar was collected as described (Carter *et al.*, 1999). Floral stages were classified as described (Koltunow *et al.*, 1990; Carter and Thornburg, 2004). Ammonium sulfate precipitation as described (Carter and Thornburg, 2000) yielded a crude nectar protein preparation.

N-terminal sequencing

Crude nectar protein (100 μ g) was subjected to 12% SDS PAGE (Laemmli, 1970), briefly stained with Coomassie Blue R-250 and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane. The bands corresponding to the *NEC2* and *NEC3* proteins were excised from the PVDF membrane and sequenced on an Applied Biosystems 477A protein sequencer/120A analyzer using sequential Edman degradation at the Iowa State University Protein Facility.

Cloning of the *NEC3* cDNA

Degenerate oligonucleotides, NecII-REV and NecIII-FOR, derived from the N-terminal amino acid sequences of *NEC2* and *NEC3* were: 5'-GGNGARGTNGAYGAYGA-3' and 5'-NGGYTTRTARTCYTTYTGNA-3' respectively, where R = G + A, Y = T + C, and N = G + A + T + C. These oligonucleotides were used for degenerate RT-PCR to generate a partial cDNA. RNA was isolated (Chomczynski and Sacchi, 1987) and reverse transcribed using the SMART cDNA library synthesis kit (Clontech, Palo Alto,

CA, USA). PCR was performed according to standard protocols (Erlich, 1989).

A Stage 12 (mature) nectary cDNA library (Carter and Thornburg, 2004) was screened using the partial *NEC3* cDNA as a probe. Strongly hybridizing plaques were brought through two rounds of screening. Positive plaques were transduced into *E. coli* (BM25.8, Clontech, Palo Alto, CA, USA). Resultant plasmids were fully sequenced in both directions at the Iowa State University DNA Sequencing and Synthesis Facility.

MALDI-TOF mass spectrometry

Crude nectar protein (100 μ g) was subjected to 10% SDS PAGE and briefly stained with Coomassie Blue G-250. The corresponding *NEC3* band was excised, destained and digested with trypsin *in situ*. Trypsinized peptides were then eluted from the gel and subjected to MALDI-TOF mass spectrometry (Wang *et al.*, 2000). Masses were collected over a range of 600–4000 Daltons.

RT-PCR determination of NEC3 expression patterns

RNA was isolated from various tissues according to Chomczynski and Sacchi (1987). One microgram of total RNA was used for first strand cDNA synthesis according to standard methods using oligo-dT (Ausubel *et al.*, 1987). Internal oligonucleotides, NecII-FOR (5'-AATTGCATTGGCACACACC-3') and N3-R1 (5'-AAATCCATCATGAA-CAGCTTC-3'), were designed and standard RT-PCR was performed. The plasmid, pRT521, which contained the full-length *NEC3* cDNA, was used as a positive control. PCR reactions were analyzed by 1% TAE-agarose electrophoresis.

Enzyme assays

Carbonic anhydrase assay

Crude nectar (50 μ l) was subjected to 10% native PAGE. An in-gel carbonic anhydrase assay was performed (Edwards and Patton, 1966). Activity bands were photographed and excised from the gel. Gel pieces were then incubated at 20 °C for 15 min in 1X SDS PAGE buffer, boiled for 5 min and then subjected to standard 12% SDS PAGE (Laemmli, 1970). After electrophoresis, the gel was stained with Coomassie Blue R-250 and photographed.

Monodehydroascorbate reductase assay

For in-gel monodehydroascorbate reductase (MDHR) assays, crude nectar (50 μ l) was subjected to 10% native PAGE and stained (Kaplan and Beutler, 1967). Activity bands were photographed and excised from the gel. Gel pieces were incubated at 20 °C for 15 min in 1X SDS PAGE buffer, boiled for 5 min and then subjected to standard 12% SDS PAGE (Laemmli, 1970). After electrophoresis, the PAGE gel was stained with Coomassie Blue R-250 and photographed.

Ascorbate determination in tobacco nectar

The presence of ascorbate was determined using both a DCIP reduction assay (Peller, 1998) as well as thin-layer chromatography analysis (Bolliger and König, 1969).

Results

Attempts to identify and characterize the tobacco *NEC2* and *NEC3* proteins were undertaken to evaluate possible functions of these proteins in nectar. Our strategy to characterize these nectarins was to isolate the proteins and subject them to N-terminal sequencing analyses. Based upon N-terminal sequences, efforts were initiated to isolate corresponding genes.

Identification of NEC2 and NEC3 as a dioscorin-like protein

N-terminal sequencing

A preparation of crude nectar proteins was separated by 12% SDS PAGE and blotted onto a PVDF membrane. The bands corresponding to *NEC2* and *NEC3* were excised and subjected to N-terminal sequencing. The *NEC2* N-terminal sequence was determined to be ^{G/V}VSNLRILQK-DYKP while *NEC3* was GEVDDESEF^{S/F}Y. BLAST searches (Altschul *et al.*, 1990) identified these sequences as a dioscorin-like protein. The *NEC3* N-terminal sequence showed identity with the N-terminus of dioscorin; however, the *NEC2* N-terminal sequence had homology to a region of dioscorin that was 48 amino acids downstream of the mature dioscorin N-terminus. These results suggested that *NEC2* might be a degradation product of *NEC3*.

Isolation and characterization of the NEC3 cDNA
Based upon these peptide sequences, we developed a strategy for the isolation of the full-length *NEC3* cDNA (Figure 2, panel A). Degenerate oligonucleotides based upon the N-terminal sequences of *NEC2* and *NEC3* were designed for PCR amplification of a partial cDNA. Total RNA was isolated from mature nectaries (Stage 12) and reverse transcribed with the SMART cDNA library construction kit. Starting with the first strand cDNA, PCR using degenerate oligonucleotides was used to generate a partial clone encoding the *NEC3* cDNA. This fragment was blunt-end cloned into the *HincII* site of pUC8 to generate the clone pRT518. This partial cDNA was 185 nucleotides in length and corresponded to the first 61 amino acids of the mature *NEC3* protein. The sequence of pRT518 is shown in Figure 2, panel B. This figure also shows the translated amino acid sequence of pRT518 aligned with the N-terminal amino acid sequences of *NEC2* and *NEC3*.

This partial *NEC3* cDNA was used to screen a mature (Stage 12) nectary cDNA library (Carter and Thornburg, 2004) and the 5' end of

six clones were sequenced and found to be identical. A single clone was designated as pRT521 and completely sequenced. This sequence was deposited in GenBank as accession #AF492468.

The isolated *NEC3* cDNA encodes a protein of 274 amino acids. The PSORT analysis tool (Nakai and Kanehisa, 1992) predicted a 25 amino acid N-terminal signal peptide that correctly identified the mature N-terminus of *NEC3*. The full length *NEC3* protein has a predicted mature mass of 28,536 Da and a pI 5.79. The truncated form of the protein, *NEC2*, has a predicted molecular weight of 23,079 Da and a pI of 8.60. The observed sizes of the *NEC2* and *NEC3* proteins were 35 and 40 kD, respectively, by SDS PAGE analysis. The differences between observed and expected masses may be due to the presence of *N*-glycosylation. There are three potential sites of *N*-glycosylation at Asn-89, Asn-112 and Asn-262 in the translated *NEC3* cDNA. It is interesting to note that none of the predicted tryptic peptides containing these glycosylation sites were identified in the MALDI-TOF MS data set (see below),

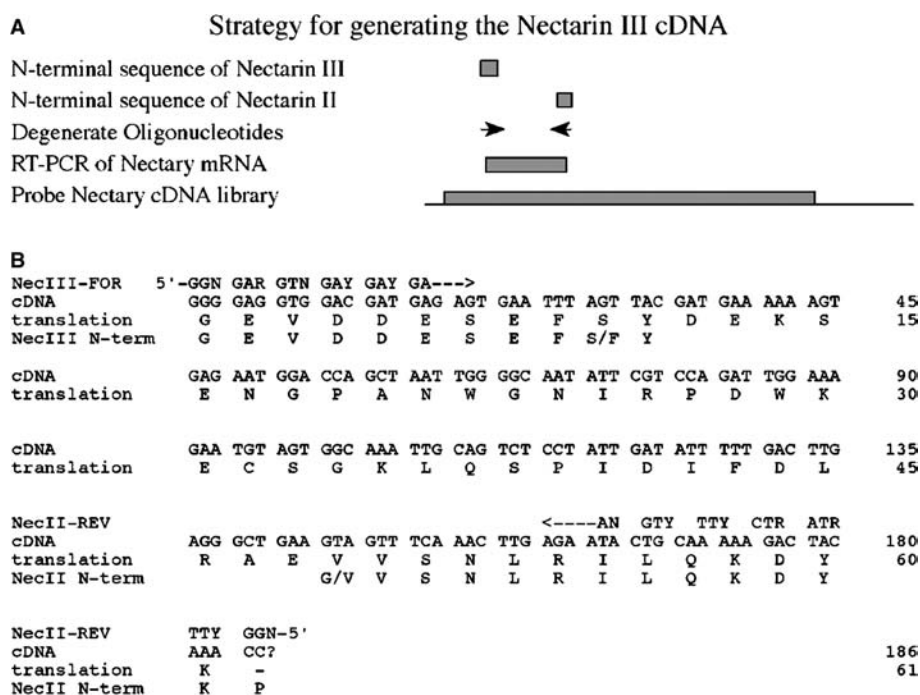


Figure 2. Cloning of the *NEC3* cDNA. (A) Strategy for isolating the *NEC3* cDNA. (B) Sequence of the partial *NEC3* clone obtained by RT-PCR. Presented are: the sequences of the oligonucleotides NecIII-FOR and NecII-REV used for amplification of the cDNA; the nucleotide sequence of the partial cDNA, pRT518; the translated amino acid sequence derived from the cDNA; and the N-terminal amino acid sequences of the *NEC2* and *NEC3* proteins.

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MRMAAITKML FISFLFLSSV FLARSGEVDD ESEFSYDEKS ENGPANWGNL 50
(NecIII) XEVDD ESEFSYDEKS ENGPA

RPDWHECSGK LQSPIDIFDL RAEVVSNLRI LQKDYKPSNA TLLNRGHDIM 100
(NecII) XVSNLRI LQKDYKPS

LRLDDGGYLK INETQYQLKQ LHWHTPSEHT INGERFNLEA HLVHESNNGK 150

FVVGIVYEI GLWDPDFLSM IENDLKVPAN KKGIERGIGI IDPNQIKLDG 200

KKYFRYIGSL TTPPCTEGVV WIIDRKVKTV PRRQINLLQE AVHNGFETNA 250

RPTQPENERY INSTSHSFGI EKQQ
274

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Figure 3. *NEC3* cDNA translation and peptides identified by MALDI-TOF fingerprinting analysis. Presented are: the translated sequence of pRT521; peptides identified by MALDI-TOF fingerprinting (shaded in gray) and the corresponding N-terminal sequence of *NEC2* and *NEC3*. The signal peptide is underlined and sites of N-glycosylation are indicated by dots.

suggesting that all three of these sites may be glycosylated in the mature *NEC3* protein.

MALDI-TOF MS analysis of the *NEC3* protein

To confirm that pRT521 encoded the *NEC3* protein, we performed a mass spectrometric peptide fingerprinting analysis of the mature *NEC3* protein. From this analysis we identified 9 peptides that matched the predicted masses from the translated sequence of *NEC3* clone. These peptides covered 41.4% of the amino acid sequence of the mature *NEC3* protein. Figure 3 and Table 1 show the peptides, which were identified. Thus, we conclude that the *NEC3* cDNA does indeed encode the *NEC3* protein isolated from the nectar of ornamental tobacco.

Enzymatic activities of *NEC3*

BLAST analysis of the full-length cDNA identified *NEC3* as a dioscorin-like protein. Dioscorin is the major soluble storage protein from yam and has a monomer mass of 28 kDa (Conlan *et al.*, 1995; Hou *et al.*, 1999a, b, 2001). This protein has been associated with several functions including carbonic anhydrase, MDHR, trypsin inhibitor, and has direct antioxidant activities (Hou *et al.*, 1999a, b, 2001). A large family of dioscorin/carbonic anhydrase-like proteins is found throughout the plant kingdom. Nucleotide sequences identified by BLAST analysis having high identity ($<e^{-40}$) to the *NEC3* cDNA were analyzed using the GCG program 'pileup'. These sequences fell into 4 clades

Table 1. MALDI-TOF peptide analysis of the mature *NEC3* protein.

	Mass		Sequence	Range ^a
	Observed	Predicted		
1	841.46	841.43	GHDIMLR	96–102
2	880.61	880.44	LDDGGYLK	103–110
3	887.51	887.49	AEVVS NLR	72–79
4	1167.69	1167.67	GIGI DPNQIK	187–197
5	1316.73	1316.72	LQSPIDIFDLR	61–71
6	1648.67	1648.65	GEVDD ESEFSYDEK	26–39
7	1708.81	1708.84	FNLEAHLVHESNNGK	136–150
8	1840.90	1840.87	SENGPANWGNIRPDWK	40–55
9	1941.89	1941.93	QLHWHTPSEHTINGER	120–135

^aThe amino acid ranges correspond to the full length *NEC3* amino acid sequence predicted from the *NEC3* cDNA.

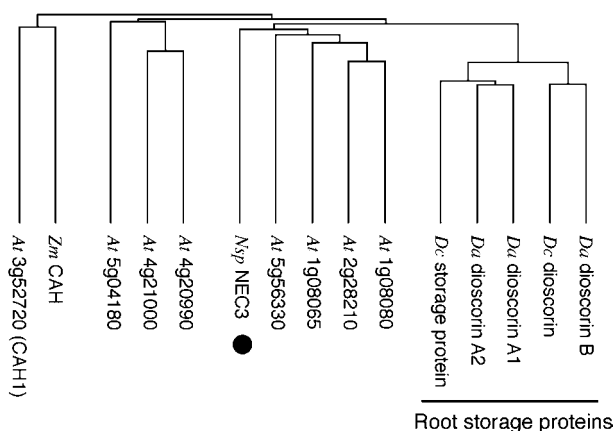


Figure 4. Alignment of dioscorin-like protein sequences. The tobacco *NEC3* is indicated by a black dot. Those sequences known to be root storage proteins from *Dioscorea* spp. form a separate clade from the other sequences. The sequences used were *At3g52720* [*A. thaliana* (*CAH1*)] (GenBank Accession #U73462), *Zea mays* PCO087467 (AY105354), *At5g04180*, *At4g21000* (NP_193832), *At4g20990* (NP_193831), *Nicotiana* sp. *NEC3* (AF492468), *At5g56330* (NP_200444), *At1g08065* (NP_172285), *At2g28210* (NP_180388), *At1g08080* (NP_172287), *Dioscorea cayenensis* storage protein (X76187), *D. alata* dioscorin A2 (AF245019), *D. alata* dioscorin A1 (AF242551), *D. cayenensis* dioscorin (S57767), *D. alata* dioscorin B (AF243526).

(Figure 4). The first clade contains the Arabidopsis carbonic anhydrase (*AtCAH1*) and a maize homologue. A second clade contains three *Arabidopsis thaliana* genes (*At4g20990*, *At4g21000*, and *At5g04180*). The functions of these genes have not been examined. A third clade contains the tobacco *NEC3* cDNA and four additional Arabidopsis genomic clones (*At1g08065*, *At1g08080*, *At2g28210*, and *At5g56330*). Finally, a fourth clade consists of the dioscorin root storage proteins of *Dioscorea alata* and *D. caryenensis*. The fact that enzymes in three different clades of this phylogenetic tree all have carbonic anhydrase

activity suggests that all of these closely related sequences may have similar activities.

Because dioscorin has several enzymatic activities, we performed experiments to evaluate the enzymatic activities of *NEC3*. To examine whether *NEC3* had carbonic anhydrase activity, we utilized the in-gel carbonic anhydrase assay (Edwards and Patton, 1966). The positively staining band (Figure 5, lane 1) was excised from the gel, re-electrophoresed on a 10% SDS PAGE, and stained with Coomassie Blue. Following staining of the SDS PAGE with Coomassie Blue R-250, the protein responsible for the activity was visualized

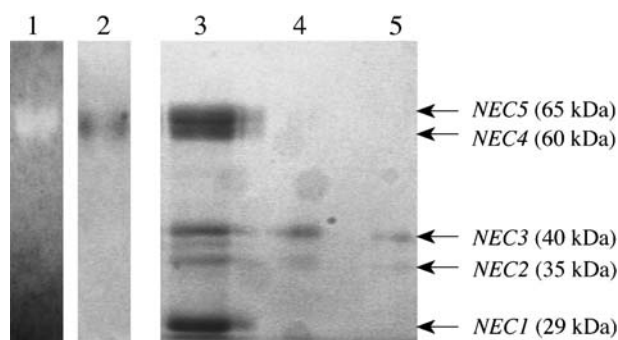


Figure 5. Identification of *NEC3* carbonic anhydrase and MDHR activities. Fifty microliters of crude nectar was subjected to standard 10% native PAGE and stained for carbonic anhydrase activity (lane 1) or MDHR activity (lane 2) as described in Materials and methods. The activity bands were excised from the native gels in lanes 1 and 2, incubated in 1× Laemmli buffer for 15 min at 20 °C, and then boiled for 5 min. The gel slices were then reapplied to standard 10% SDS PAGE and stained with Coomassie blue R-250 (lanes 4 and 5). Lane 3: 50 μl crude nectar containing all nectarins. Lane 4: proteins present in the positively staining carbonic anhydrase activity band from lane 1. Lane 5: proteins present in the positively staining MDHR activity band from lane 2.

(Figure 5, lane 4). *NEC3* is found in the carbonic anhydrase activity band isolated from the native gel indicating that *NEC3* is responsible for the carbonic anhydrase activity in nectar. A major function of carbonic anhydrase activity in animal systems is maintenance of blood pH. To examine whether an analogous function for carbonic anhydrase occurs in tobacco nectar, the pH of freshly collected nectar was measured. Results from numerous observations indicate that nectar is stably maintained at a pH of approximately 7.

A similar experiment was set up to evaluate whether *NEC3* has MDHR activity, as does the native dioscorin. Figure 4 (lane 2) shows the electrophoresis of crude nectar proteins on a 10% native polyacrylamide gel that has been stained for MDHR activity. The positively staining band was then excised and electrophoresed on a 10% SDS PAGE and stained with Coomassie Blue. Again, these results reproducibly demonstrated that the native gel band active for MDHR activity contains *NEC3*. Thus, we conclude that *NEC3* is also responsible for the observed MDHR activity.

Finally, a trypsin inhibitor assay was also performed as described in Lee and Lin (1995). However, we observed no indications that *NEC3* contained trypsin inhibitor activity.

Tissue-specific and developmental expression

To evaluate the temporal and spatial expression of *NEC3*, the presence of *NEC3* mRNA was evaluated by RT-PCR. Total RNA was isolated (Chomczynski and Sacchi, 1987) from all major tissues as well as from nectaries at various stages of development. This RNA was reverse transcribed as in Materials and methods and PCR was then performed using 1 μ l of each RT reaction with internal oligonucleotides specific for the *NEC3* gene.

Tobacco floral development can be divided into twelve discrete stages, which were defined by Koltunow *et al.* (1990). During the process of floral development, the nectary also undergoes a series of discrete developmental stages. The initiation of nectary development occurs at a primordial floral stage while the flower bud is still quite small. By floral Stage 1, the nectary is a well-differentiated ring of cells surrounding the primordial gynoecium. The filling stage of nectary development is characterized by an engorgement of the

nectary gland, during which the nectary characteristically enlarges several-fold, beginning at early stages and continuing through floral Stage 7 (approximately 6 days). Ripening begins about floral Stage 6 and continues through floral Stage 9. During this period, the nectary gland turns from green to bright orange due to the synthesis and accumulation of β -carotene (Barua and Thornburg, unpublished). The duration of the ripening stage is approximately 24 h. Finally, nectary maturation occurs from floral Stage 9 through floral Stage 12, during which nectar proteins begin to be synthesized. Nectar flow begins at late Stage 10, approximately 12 h prior to floral opening. Anthesis occurs at Stage 12. The duration of the maturation stage is approximately 12 h. The post-maturation stage comprises all stages that occur after anthesis and lasts as long as the flower is receptive to pollination. During this time, nectar flow continues; however, the flow is stronger at early times and weakens the longer the flower remains open. This stage can sometimes last 6–8 days if the flower is not pollinated. Successful pollination leads to fruit development, whereas unpollinated flowers eventually fall from the plant.

To follow the patterns of *NEC3* transcript expression during nectary development, we examined nectaries from Stage 2 (early floral bud), Stage 6 (immature nectary, filling stage), Stage 8 (immature nectary, mid-maturation stage), Stage 10 (immature nectary, early maturation stage, pre-secretory), Stage 12 (mature nectary, secretory stage) and post-fertilized (PF) flowers. Figure 6, panel A shows that *NEC3* transcripts are expressed at low levels in Stage 2 nectaries and then evenly throughout the subsequent stages of nectary development. Following fertilization *NEC3* transcript expression is abolished.

To determine spatial expression patterns of the *NEC3* transcripts, all major floral and vegetative organs were also examined by RT-PCR (Figure 6, panel B). *NEC3* is expressed most strongly in the nectary gland; however, in addition to the nectary expression, *NEC3* is also expressed at lower levels in the ovary, style, stigma, floral tube, and at low levels in the anthers/filaments. *NEC3* was not expressed in other parts of the plant, nor was it expressed in other floral organs.

NEC3 expression patterns vary significantly from that of *NEC1* and *NEC5*. *NEC3* transcripts are found in Stage 2 through Stage 12 nectaries as

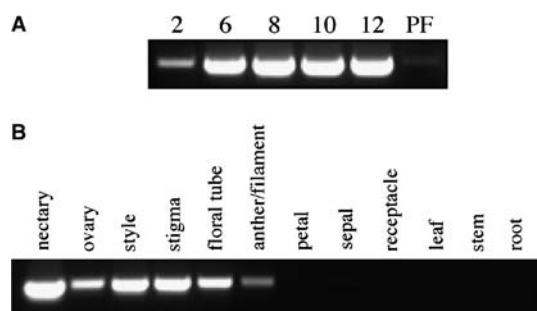


Figure 6. *NEC3* expression patterns. Whole RNA was isolated from the indicated tissues and RT-PCR was performed as in Materials and methods. (A) Temporal expression of *NEC3* transcript in ornamental tobacco nectaries. The floral stages assayed for RNA are indicated above each lane. PF = post-fertilization. (B) Spatial expression of *NEC3* transcript in ornamental tobacco nectaries. The floral organ or vegetative tissue that was the source of the RNA is indicated above each lane.

well as in various floral organs (Figure 6). *NEC1* and *NEC5* are expressed solely in nectary tissues and only at times when nectar is flowing (Carter *et al.*, 1999; Carter and Thornburg, 2003, 2004). Because of these differences, we conclude that there must be multiple molecular mechanisms responsible for expression of genes in the nectary gland. Unfortunately, the 5' flanking region of the *NEC3* gene has not been isolated, so promoter comparisons cannot yet be performed.

Discussion

We have previously described a small set of proteins (nectarins) that are expressed in the floral nectar of ornamental tobacco. *NEC1*, is a manganese containing germin-like SOD (Carter *et al.*, 1999; Carter and Thornburg, 2000). The *NEC1* promoter has been isolated and reporter constructs demonstrate that the gene is expressed exclusively in actively secreting nectary tissue (Carter and Thornburg, 2003). *NEC5* is a flavin-containing berberine bridge enzyme-like protein with glucose oxidase activity (Carter and Thornburg, 2004). We have now identified the *NEC2* and *NEC3* proteins, determined enzymatic activities, isolated the corresponding cDNA and resolved patterns of expression.

Comparison of the N-terminal sequences of the *NEC2* and *NEC3* proteins with the *NEC3* cDNA sequence indicates that *NEC2* is a degradation

product of *NEC3*. The site of cleavage (Glu-73) removes 48 amino acids from the mature *NEC3* protein. The removal of this protein fragment appears to knock out both carbonic anhydrase and MDHR activity because the active complexes contained only the *NEC3* protein and not the *NEC2* protein. Modeling (Peitsch, 1995, 1996; Guex and Peitsch, 1997) of *NEC3* on the structure of *Neisseria gonorrhoeae* carbonic anhydrase (1KOQA; Huang *et al.*, 1998) reveals that this glutamine residue is in a loop and is surface exposed. The fragment that is removed makes intimate contact with both the C-terminal region of the protein as well as with two internal β -loops, so removal of this fragment would significantly alter the structure of the *NEC3* protein. Nothing is known about the processing activity responsible for this cleavage of the *NEC3* molecule, nor do we know whether the proteolytic processing occurs prior to secretion or in nectar; however, to date, we have not identified any proteolytic activities in nectar.

Enzymatic activities of *NEC3*

NEC3 was identified as a dioscorin-like protein. Dioscorin has been associated with several functions including carbonic anhydrase, MDHR, and trypsin inhibitor activities (Hou *et al.*, 1999a, b 2001). In this study, *NEC3* was found to contain carbonic anhydrase and MDHR activities (Figure 5).

NEC3 belongs to the α -class of carbonic anhydrases (Tripp *et al.*, 2001) and has identity with the human carbonic anhydrase II (Kannan *et al.*, 1972). The enzymatic activity of human carbonic anhydrase II has been well studied (for review see Christianson and Cox, 1999). In this enzyme, a single tetrahedral-coordinate zinc molecule is liganded by three histidine residues (His-94, His-96, and His-119) and a hydroxide ion. The zinc-bound hydroxide ion donates a hydrogen bond to Thr-199, which in turn donates a hydrogen bond to Glu-106. A hydrophobic binding pocket located proximally to the zinc-binding site, which serves to accept the substrate, CO₂, is lined by hydrophobic amino acids (Val-121, Val-143, and Leu-198). The three histidine rings that coordinate the zinc ion are further stabilized by second-shell liganding residues (Gln-92, Glu-117 and the backbone carbonyl of Asn-244). In the catalytic process, the substrate,

CO₂, binds to the hydrophobic pocket, followed by a nucleophilic attack of the zinc-bound hydroxyl anion on the CO₂. The hydrogen bound hydroxyl side chain of Thr-199 serves to stabilize the transition state of CO₂ hydration and it also destabilizes the binding of bicarbonate, thereby facilitating product release. A water molecule from solvent then replaces the bound bicarbonate. To re-establish the catalytic state of the enzyme, a hydrogen ion must be removed from the zinc-bound water molecule by proton diffusion across a hydrogen-bonded solvent network ultimately to His-64. Upon protonation, His-64 undergoes a rate limiting conformational change that displaces the proton away from the solvent network.

Comparisons of *NEC3* with the human carbonic anhydrase structure reveal that each of these active site side chains is conserved in *NEC3* (see Table 2). Because of this high degree of conservation between both the structure and the conserved active site residues, we speculate that the *NEC3* carbonic anhydrase activity may be mediated via the same mechanism that has been previously established for the human enzyme.

Functions of NEC3 in nectar

The physiological roles of *NEC3* in nectar can be inferred from its biochemical activities. The carbonic anhydrase activity of *NEC3* may function to buffer the pH of nectar. We have evaluated the pH of ornamental tobacco nectar by direct measurement and found that it is stably maintained near a pH of 7. Stabilizing the pH of nectar would ensure

that the biochemical properties of nectar proteins function efficiently. It would also provide a pH-balanced meal for visiting pollinators. It is interesting to note that plants use the same mechanism (carbonic anhydrase) to buffer nectar that animals use as the primary buffer of blood.

A need for active pH maintenance in nectar may arise from two distinct chemical reactions that occur in nectar (Figure 7). First, *NEC1* is a SOD (Carter and Thornburg, 2000). The overall reaction of SOD consumes hydrogen ions ($2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$). Second, floral nectar contains transition metal ions, notably Cu⁺² and Fe⁺³ (Heinrich 1989). It is well known that hydrogen peroxide is unstable in the presence of metal ions, breaking down to form hydroxyl free radicals (OH[•]) (Rowley and Halliwell, 1983; Halliwell and Gutteridge, 1999). Fortunately, nectar also contains ascorbate, which reduces these free radicals to form hydroxide anions (OH⁻) resulting in the production of monodehydroascorbate (MDH) (Schneider *et al.*, 1988; Rose, 1990). Each of these reactions would cause tobacco nectar to become basic, which would likely affect pollinator feeding. Therefore, we hypothesize that one role of *NEC3* in nectar would be to produce a buffering system in nectar that would counteract the propensity for tobacco nectar to become basic.

We also propose a role of *NEC3* in maintaining ascorbate concentrations in nectar (Figure 7). If there were no way to regenerate ascorbate levels in nectar, the oxidation of ascorbate to MDH in the process of scavenging hydroxyl radicals would

Table 2. Conserved active site residues between human carbonic anhydrase and *NEC3*.

Function	Amino acid position	
	Human carbonic anhydrase	<i>NEC3</i>
Coordinates zinc atom	His-94	His-122
	His-96	His-124
	His-119	His-141
Hydrogen bond-relay	Thr-199	Thr-211
	Glu-106	Glu-128
Hydrophobic binding pocket	Val-121	Val-143
	Val-143	Val-153
	Leu-198	Leu-210
	His-64	His-97
Shuttle residue	His-64	His-97
Second shell, stabilizes and orients His 94	Gln-92	Gln-120
Second shell, stabilizes and orients His 96	Backbone carbonyl of Asn-244	Unidentified
Second shell, stabilizes and orients His 119	Glu-117	Glu-139

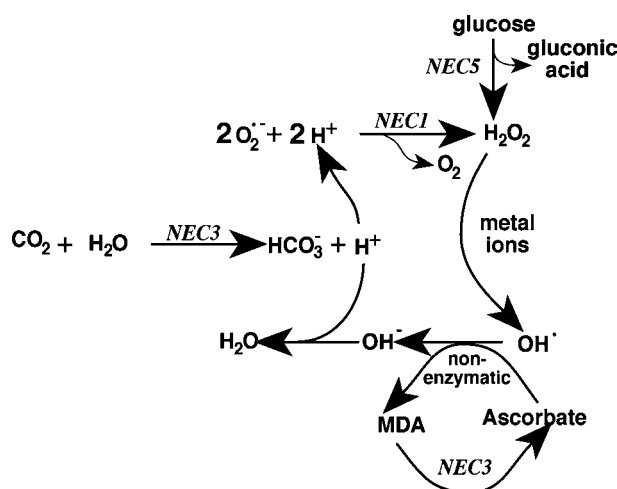


Figure 7. Proposed function of NEC3 in nectar. NEC3 = Nectarin III, NEC1 = Nectarin I, NEC5 = Nectarin V, MDA = monodehydroascorbate.

eventually lead to a significant decline in nectar ascorbate levels. We have found that ascorbate is present in tobacco nectar at a concentration approximately one fourth that of hydrogen peroxide. Hydrogen peroxide is present in nectar at concentrations up to 4 mM (Carter and Thornburg, 2000) and for this study we have measured the average levels of ascorbate in the nectar of our ornamental tobacco plants at 0.97 mM. Further, ornamental tobacco flowers often secrete nectar for up to 8–10 days at a time if the flower remains unpollinated. We propose that the MDHR activity of NEC3 functions to convert MDH back into ascorbate, thereby protecting floral tissues from the possibility of damage from free radical attack. Interestingly, both dioscorin and *SABP3* (a tobacco chloroplastic carbonic anhydrase/salicylic acid binding protein) were recently described as having direct antioxidant activities (Hou *et al.*, 2001; Slaymaker *et al.*, 2002). Whether or not NEC3 shares this activity is currently under examination; however, such an activity in nectar would also protect reproductive tissues from oxidative damage.

The cumulative identification and characterization of nectar proteins demonstrates that plant nectars contain significant and unique biochemistry (Carter and Thornburg, 2000, 2004; Thornburg *et al.*, 2003; this report). Nectar is not merely secreted from the plant and left untended, but is actively involved in the defense of the floral gynoecium (Thornburg *et al.*, 2003). The proteins in

nectar likely serve to actively maintain nectar in an axenic state, while at the same time protecting the plant from the potentially damaging products used to generate a microbial inhospitable environment.

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