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The nectary-specific pattern of expression of the tobacco Nectarin I promoter is regulated by multiple promoter elements†

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†*Dedicated in memoriam to Dr. Jubran Wakim, Department of Chemistry, Middle Tennessee State University*

Key words: defense, germin-like protein, nectar, nectary, *Nicotiana*, ovary, SOD, superoxide dismutase

Abstract

The major protein secreted into the nectar of tobacco plants (Nectarin I) is a germin-like protein that has superoxide dismutase activity. We have isolated the gene encoding Nectarin I (*NECI*) and analyzed the expression of a chloramphenicol acetyl transferase (*CAT*) marker gene driven by its promoter in transgenic plants. Transgenic plant lines that expressed the CAT gene under control of the full-length *NEC1* promoter showed high levels of CAT expression in mature floral nectaries. Tissue specificity of *NECI-CAT* expression demonstrated that the construct was expressed uniquely in nectaries with a small level of expression in ovary. Further, analysis of its temporal expression showed that the construct is expressed uniquely during those times when nectar is actively being secreted from flowers. An examination of the transcription start site verified that the initiation site of the *NECI-CAT* mRNA in transgenic plants is identical with that of the native gene *in vivo*. Two promoter deletion constructs were also prepared and analyzed. Analysis in transgenic plants revealed that the nectary-specific expression is the result of multiple promoter elements and suggests that nectar secretion and flower opening may be coordinately regulated.

Abbreviations: NEC1 – Nectarin I, SOD – superoxide dismutase, CAT – chloramphenicol acetyl transferase

Introduction

Many plants offer floral nectar as a reward to attract insect, avian, or mammalian pollinators. The secretion of nectar is usually under developmental control beginning just prior to flower opening. After pollination, the nectar is frequently resorbed (Burquez and Corbet, 1991). In addition, nectar secretion increases as the flower is visited by pollinators (Smith *et al.*, 1990).

The composition of nectar has been widely studied. Nectar is an aqueous combination of a significant number of solutes. Chief among these are sucrose, glucose, and fructose. Other carbohydrates have also been identified in nectars of some flowers (Baker and Baker, 1981). A relationship exists between the sugar composition of nectar and the amount of vascular tissue underlying the nectary (Esau, 1977). If phloem makes up most of the vascular tissue, the nectar may contain up to 50% sugar. Conversely, if xylem predominates, the sugar content may fall to as little as 8% (Frey-Wyssling, 1955).

Most nectars also contain amino acids (Baker and Baker, 1973). All 20 of the normal amino acids found in protein have been identified in various nectars, with alanine, arginine, serine, proline, glycine, isoleucine, threonine, and valine being the most prevalent. A wide

variety of other organic substances have been reported in nectar (Baker and Baker, 1975; Cabras *et al.*, 1999; Deinzer *et al.*, 1977; Ecroyd *et al.*, 1995; Ferreres *et al.*, 1996; Griebel and Hess, 1940; Rodriguez-Arce and Diaz, 1992; Roshchina and Roshchina, 1993; Vogel, 1969).

In addition, many dicotyledenous plants also secrete a limited array of proteins into nectar (Carter *et al.*, 1999). We have designated these proteins nectarins. Recently, we examined the expression of the major tobacco nectar protein, Nectarin I (Carter *et al.*, 1999). As determined by western blotting, the expression of Nectarin I is limited to nectary and ovary tissues and is expressed only at times when nectar is actively being secreted (Carter *et al.*, 1999). Subsequently, we have purified tobacco Nectarin I to homogeneity and identified its function as a manganese superoxide dismutase ($2O_2^{\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$). Hydrogen peroxide was found at levels up to 4 mM in tobacco nectars (Carter and Thornburg, 2000). Thus, Nectarin I may generate high levels of hydrogen peroxide in tobacco nectar. Indeed, this finding suggests that the nectar proteins may play a defensive role in protecting the ovary and developing seeds. In this study we have prepared chimeric constructs linking the *N. plumbaginifolia* Nectarin I promoter to a chloramphenicol acetyl transferase marker gene and analyzed their expression in transgenic plants to understand the Nectarin I promoter.

Materials and methods

Materials

[^{14}C]-chloramphenicol, 60 mCi/mmol, was purchased from New England Nuclear/Dupont (Boston, MA). All other materials were of reagent grade and were obtained either from Sigma Chemical Co. (St. Louis, MO) or Fisher Chemical Co. (Pittsburgh, PA).

Preparation of NECI-CAT Constructs

The bacteriophage lambda clone containing the *Nicotiana plumbaginifolia* Nectarin I gene (Carter *et al.*, 1999) was digested with *Bgl*II. A 3.7-kb fragment that corresponded to the Nectarin I gene was isolated by agarose gel electrophoresis and was subsequently cloned into the *Bam*HI site of pUC8 to make the vector, pRT454. The entire 3,685 nucleotide insert of pRT454 was sequenced (GenBank Accession #AF132671), and PCR oligonucleotides were designed (see Table 1) to amplify 1.35 kb of 5′ flanking regions of the Nectarin I gene. Following PCR amplification the PCR product was digested with *Hin*dIII and *Xba*I, and the fragment was cloned into *Hin*dIII/*Xba*I-digested pUC119 to make the vector, pRT460. The insert of this vector was confirmed by DNA sequencing. It contained 1.2 kb of the Nectarin I promoter (the *Hin*dIII digestion removed approximately 150 nt from the very 5′ end of the promoter). Subsequently, the Nectarin I promoter was gel purified after *Hin*dIII/*Xba*I digestion of pRT460 and was cloned into the *Hin*dIII/*Xba*I sites of the plant transformation vector, pRT190 (Park and Thornburg, 1996) to make pRT461. The full length promoter contains sequences from −1187 to +60 of the Nectarin I gene (AF132671). Similar constructs were prepared with two promoter deletions. pRT500 contains sequences from −916 to +60 of the Nectarin I gene in the vector pRT190. pRT502 contains sequences from −275 to +60 of the Nectarin I gene in the vector pRT190. The vector, pRT190, was derived from pBI101.1 and features a promoterless chloramphenicol acetyl transferase gene linked to the strong *pin2K* potato Proteinase Inhibitor II terminator (An *et al.*, 1987, Thornburg *et al.*, 1987). The sequence of the *NECI-CAT* gene fusion was confirmed in all constructs by sequencing through the promoter-CAT gene junction.

Plant Transformation

The plant transformation vectors were transferred into *Agrobacterium tumefaciens* LBA4404 (An *et al.*, 1986), and the resulting *Agrobacterium* strains were used to transform *Nicotiana tabacum* cv. Xanthi plants to kanamycin resistance (Thornburg *et al.*, 1987). Plants were maintained in tissue culture with 14hr days and 10hr nights until they were approximately 10 cm tall, when they were transferred to soil. Plant growth continued until maturity in the greenhouse.

Tissue Isolation

Isolation of floral tissues was as previously described (Carter *et al.*, 1999). If not used immediately, tissues were maintained at −20 °C until use.

Chloramphenicol Acetyl Transferase Assay

Extracts of transformed tobacco tissues were prepared by homogenizing the tissues in a mortar and pestle with the addition of 1 volume/g fresh weight of homogenization buffer (Kernan and Thornburg, 1989).

Table 1. Oligonucleotides used in these studies

Name	Sequence	Purpose	
1	5′-cap SMART IV oligo	5′-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3′	Ligation at the 5′ end of the first strand cDNAs
2	5′-PCR primer	5′-AAGCAGTGGTATCAACGCAGA-3′	Templates for PCR of cDNAs
3	3′ PCR primer	5′-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)$_{30}$NN-3′	
4	oligo 1	5′-GGAAGCTTGGATCTCTGGGACCT-3′	PCR amplification of the *NECI* promoter
5	oligo 2	5′-TATCTAGACCACCAGCTCAAG-3′	
6	CAT-forward	5′-GTCTTTCATTGCCATACGG-3′	PCR amplification of the 5′ end of the *NECI-CAT* mRNA
7	CAT-reverse	5′-CAAAATGTTCTTTACGATGCC-3′	

Following homogenization, the lysate was centrifuged for 5 min at 10 000× g at 4 °C, and the supernatant was recovered for protein analysis by the method of Bradford (1976). A volume from each extract containing 25 μg of protein was assayed for CAT activity as described (Gorman *et al.*, 1982). Following TLC analysis, the activity of the CAT protein was visualized by exposure to X-ray film. After development of the X-ray films, the plastic-backed TLC plates were overlaid onto the exposed films and the radioactive spots corresponding to the labeled chloramphenicol acetates and unreacted chloramphenicol were cut out. Radioactivity was measured in a Packard liquid scintillation counter. The data are expressed as percent conversion of chloramphenicol into chloramphenicol acetates.

For quantitative CAT assays, all extracts in a given experiment contained the same amount of protein and were diluted so that the most concentrated sample was still in the linear range of the CAT assay as empirically determined by kinetic assays.

Transcription Start Site Analysis

Total RNA was isolated from the nectaries of Tr461.3 flowers at stage 12 of development by the method of Chomczynski and Sacchi (1987). One microgram of this RNA was reverse transcribed with PowerScript reverse transcriptase, and ds cDNA was amplified using the SMART cDNA kit according to the manufacturer's protocols (Clontech, Palo Alto, CA). Following preparation of the cDNAs, the ends were polished with Klenow Fragment (Ausubel *et al.*, 1992), ethanol precipitated and circularized via ligation. An inverse PCR method using oligonucleotides specific to the CAT gene was employed to identify the transcription start site. DNA sequencing was performed at the Iowa State University Nucleic Acid facility using the Applied Biosystems Prism Dye-deoxy Cycle Sequencing Kit. The reactions were run on an Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer Corp., Norwalk, CT).

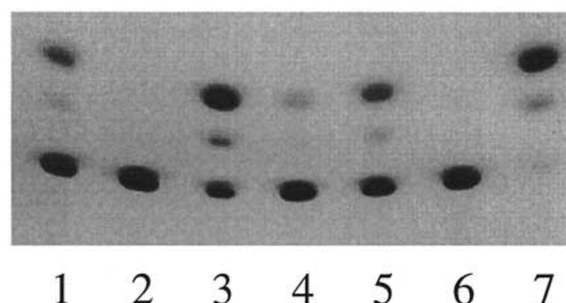

Figure 1. Assay of chloramphenicol acetyl transferase activity in nectary tissue (Stage 12) of five independent Tr461 lines. Extracts of plant tissues containing 25 μg of protein were assayed for chloramphenicol acetyl transferase as described in Materials and Methods. Lane 1, Tr461.1; lane 2, Tr461.2; lane 3, Tr461.3; lane 4, Tr461.4; lane 5, Tr461.5; lane 6, negative control containing wild-type *N. tabacum* cv Xanthi; lane 7, positive control containing 45 units of *E. coli* chloramphenicol acetyl transferase

Results and Discussion

The *NECI-CAT* construct (pRT461) was prepared as described in Materials and Methods and used to transform *N. tabacum* cv. Xanthi plants to kanamycin resistance. Five lines of transgenic plants were produced in these studies. The presence of the transgene was con-

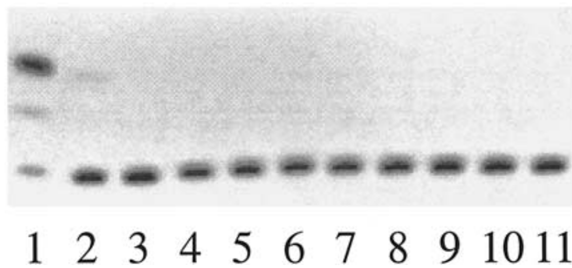

Figure 2. Organ specificity of expression of the *NECI-CAT* construct, pRT461. Extracts of various plant tissues containing 25 μg of protein were assayed for chloramphenicol acetyl transferase as described in Materials and Methods. Lane 1, nectary; lane 2, ovary; lane 3, style; lane 4, stigma; lane 5, anthers/filaments; lane 6, floral tube; lane 7, petals; lane 8, sepals; lane 9, receptacle; lane 10, leaf; lane 11, root.

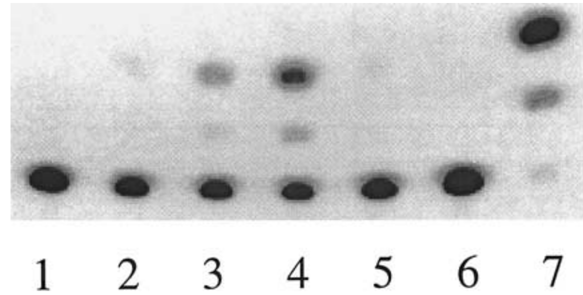

Figure 3. Developmental expression of the *NECI-CAT* construct, pRT461. Extracts of various plant tissues containing 25 μg of protein were assayed for chloramphenicol acetyl transferase as described in Materials and Methods. Lane 1, nectary tissues from Stage 6 flowers; lane 2, nectary tissues from Stage 8 flowers; lane 3, nectary tissues The tobacco Nectarin I promoter from Stage 10 flowers; lane 4, nectary tissues from Stage 12 flowers; lane 5, nectary tissues from flowers postfertilization; lane 6, negative control containing no added tissue extract; lane 7, positive control containing 10 μg of *E. coli* chloramphenicol acetyl transferase.

firmed in each of these plants by PCR analysis (data not shown). Each of these lines was grown to maturity and flowers were isolated from each plant line at floral maturity (Stage 12). As can be seen in Figure 1, four of the transgenic plants showed good levels of CAT expression (>10% conversion of chloramphenicol into chloramphenicol acetates).

We had previously used western blots to demonstrate that the Nectarin I protein accumulates uniquely in nectar, nectary tissues and to a much lower extent in ovary (Carter *et al.*, 1999). When we examined the organ specific expression patterns of the *NEC1-CAT* construct containing the full length Nectarin I promoter (Figure 2), we observed that the CAT activity reflects this pattern. CAT activity was strongly observed only in the nectary tissue (Lane 1), and to a much lower level in the ovary (Lane 2). No CAT activity was observed in any other floral organ (Lanes 3 to 9), nor was CAT activity found in leaves, stems, or roots. When we quantitated the expression in each organ, we observed that the nectary has approximately 15-fold higher levels of expression than the ovary and at least 100-fold higher expression than any other plant organ. Therefore, we conclude that the Nectarin I promoter is strongly and uniquely expressed in nectary tissue and to a much lower extent in the ovary. All of the transgenic lines that showed nectary expression showed similar patterns of spatial and temporal specificity and varied only in the level of expression.

We also evaluated the developmental expression of the *NECI-CAT* construct. The development of tobacco flowers has been divided into 12 stages (Koltunow *et al.*, 1990). According to this scheme, flowers are fully open and mature at stage 12. Nectar secretion begins at late stage 10 or early stage 11, approximately 12 to 16 hr prior to floral maturity. Further, following fertilization of the flower, nectar secretion ceases. To evaluate the developmental expression of the Nectarin I promoter, we isolated nectary tissues from Tr461.3 flowers at a variety of stages between floral stage 6 and postfertilization. As can be seen in Figure 3, the *NECI-CAT* construct expression is undetectable at Stage 6 (lane 1), begins at Stage 8 (lane 2), is at moderate levels by Stage 10 (lane 3), and at high levels by Stage 12 (lane 4). In postfertilization flowers (lane 5) there was little CAT activity remaining. Quantitation of these different floral stages demonstrated that Stage 12 nectaries were at least 40-fold higher than stage 6 nectaries; 10-fold higher than stage 8 nectaries; 2.5-fold higher than stage 10 nectaries; and 12-fold higher than the postfertilization nectaries. Thus, the expression of the Nectarin I promoter precedes nectar secretion by 12 to 24 hours, but is most active at times when nectar is being actively secreted from the nectary tissues.

To evaluate whether the Nectarin I promoter utilizes the same transcription start site in the transgenic plants as it does in normal plants, we used a SMART cDNA synthesis method to isolate the full-length 5′ untranslated region of the *NECI-CAT* construct and a full-length cDNA encoding the native Nectarin I. Initially we submitted the Nectarin I promoter for on-line analysis of the transcription start site using the Neural Network Promoter Prediction tool at "http://www.fruitfly.org/seq_tools/promoter.html". This analysis predicted that nucleotide 1272 of the promoter as the transcription start site. The sequence

Table 2. Regions of identity between the Tobacco Nectarin I promoter and the Petunia NecI promoter

	GenBank Accession	Sequence	Location
myb Consensus		N M A C C W A M C[a]	
Tobacco Nectarin I	AF132671	T C A C C T A A C	−899 to −891[b]
Petunia NecI	AX006361	T C A C C T A A A	400 to 408[c]
Petunia NecI	AX006361	T C A C C T A A A	1621 to 1629[c]

[a]where M = $^{A}/_{C}$ and W = $^{A}/_{T}$
[b]relative to the transcription start site
[c]relative to the first nubleotide in the GenBank Accession

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NecI gene    AAAGAGCATA TATAACACCA CCAAAAAGAC GAAAAAGAGA CTCCTATTTT 1050
NecI gene    GAAGCAAGGC TTTTCTTTAT TTGGTATAGT ATTTTGACCA CTCTATTTAA 1100
NecI gene    TTTCTTCATC TCACGTCAAA TTTAAGTCTA GCAATACTCA TTTACTCCTC 1150
NecI gene    GCACATATAT ATCAATGTTA TAATTCTTTT ACACAAGCAC CTAATTTCAG 1200
NecI gene    GTCAATTCCA ATACCAAATT AAAGCCTTTC AAGTTTTTCC TATAAATGAA 1250

                                   +1 (transcription start)
                                   |
NecI gene    GCCACTCCAA TTTCTCTGAG CATCGTCAAA TTAATTAAAG AGTAATATTA 1300
NecI cDNA                           ATCGTCAAA TTAATTAAAG AGTAATATTA 29
transgene                           ATCGTCAAA TTAATTAAAG AGTAATATTA 29

                                                         NECI -->
NecI gene    ACGAGTTCTT CTTCTCCTTG AGCTTGTGGT CACCATATTA TGGCTGCCTT 1350
NecI cDNA    ACGAGTTCTT CTTCTCCTTG AGCTTGTGGT CACCATATTA TGGCTGCCTT 79
transgene    ACGAGTTCTT CTTCTCCTTG AGCTTGTGGT CTAGAGGATC CTCTAGGGAA 79

NecI gene    TGGAATTAAA AGTAAGATAT TTCAAATTAT GGAGATGACG ATACTATTTT 1400
NecI cDNA    TGGAATTAAT AGTAAGATAT TTCAAAGTAT GGAGATGGCG ATACTATTTT 129
transgene    GATTGAGCTT GGCGAGATTT TCAGGAGCTA AGGAAGCTAA AATGGAGAAA 129
                                                           CAT -->
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Figure 4. Transcription start site analysis of the Nectarin I promoter. The identity of each sequence is indicated to the left of each sequence. The nucleotide position of each sequence is indicated to the right of each sequence. The *N. plumbaginifolia* Nectarin 1 gene is GenBank Accession number AF132671. The native Nectarin I cDNA is GenBank Accession number AF411917. The Tr461.3 *NECI-CAT* transgene 5′ RACE analysis is labeled Tr461.3. The positions of the ATG start codons for the Nectarin I genes and for the CAT gene are indicated in boldface type.

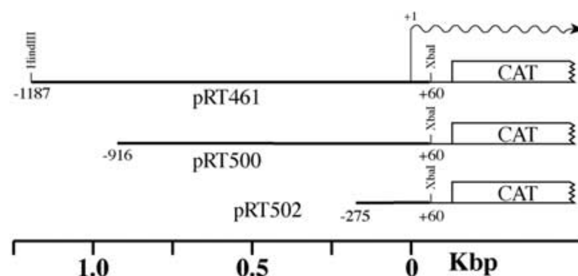

Figure 5. Nectarin I promoter deletions. The three constructs used in this study are shown. pRT461 contains sequences from −1187 to +60 of the Nectarin I gene linked to the CAT gene. pRT500 contains sequences from −916 to +60 of the Nectarin I gene. pRT502 contains sequences from −275 to +60 of the Nectarin I gene.

alignment in Figure 5 shows a portion of the Nectarin I gene and the sequences of the 5′ untranslated regions from the full-length Native Nectarin I cDNA and from the *NECI-CAT* construct. In each case, nucleotide 1272 of the promoter was identified as the transcription start site, and we conclude that the promoter in the chimeric constructs is being faithfully regulated in the transgenic plants.

The sequence of the native Nectarin I cDNA was deposited in the GenBank as accession #AF411917. The nucleotide sequence is 94% identical to that of the *N. plumbaginifolia* Nectarin I gene (Carter *et al.*, 1999). These differences result in a protein that is 96% identical at the amino acid level to the *N. plumbaginifolia* protein.

To evaluate whether promoter deletions would affect marker gene expression we prepared promoter-CAT gene fusions with two promoter deletions. pRT500 contains sequences from −916 to +60 of the *N. plumbaginofolia* gene linked to the CAT gene and pRT502 contains sequences from −275 to +60 linked to the CAT gene (Figure 5). The level of expression in pRT500 is not significantly different from that observed with the pRT461 plants. In contrast, the shortest promoter, pRT502 was not functional in the nectaries of any of the transgenic plants tested (data not shown).

To evaluate the organ specificity of the pRT500 construct, we examined floral organs for CAT activity. To our surprise, we found that by deleting 271 nucleotides from the 5′ end of the Nectarin I promoter altered the organ specificity. As is shown in Figure 6, in Tr500.2 plants, the CAT activity is present not only in the nectary, but now also appears in the petals. We also evaluated the temporal patterns of expression of CAT activity in both the nectary and in the petals. The temporal expression of CAT activity in the nectaries of the Tr500 plants was not significantly different from that of Tr461 (data not shown). Likewise, the temporal expression of CAT activity in the petals of the Tr500 plants also showed that the shorter Nectarin I promoter is still temporally regulated in a correct manner, even though the organ specificity has now been altered (compare Figure 7 with Figure 3).

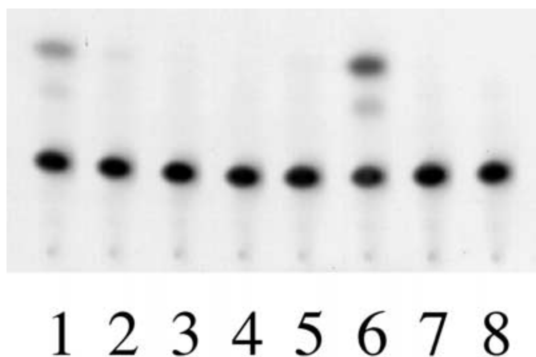

Figure 6. Organ specificity of expression of the *NECI-CAT* construct, pRT500. Extracts of various plant tissues containing 25 μg of protein were assayed for chloramphenicol acetyl transferase as described in Materials and Methods. Lane 1, nectary; lane 2, ovary; lane 3, stigma/style; lane 4, anthers/filaments; lane 5, floral tube; lane 6, petals; lane 7, sepals; lane 8, receptacle.

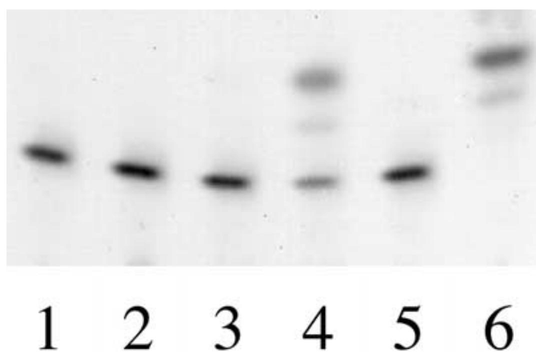

Figure 7. Developmental expression of the *NECI-CAT* construct, pRT500. Extracts of petal tissues at various stages of development containing 25 μg of protein were assayed for chloramphenicol acetyl transferase as described in Materials and Methods. Lane 1, petal tissues from Stage 6 flowers; lane 2, petal tissues from Stage 8 flowers; lane 3, petal tissues from Stage 10 flowers; lane 4, petal tissues from Stage 12 flowers; lane 5, negative control containing no added tissue extract; lane 6, positive control containing 10 μg of *E. coli* chloramphenicol acetyl transferase.

From these data, we draw two conclusions. First, that the 271 nucleotides found in pRT461 but not in pRT500 must contain an element that limits the expression of the Nectarin I promoter in the petals of the Tr461 plants. Second, promoter elements that correctly regulate the temporal specificity of the Nectarin I promoter are contained within pRT500 and must therefore be located downstream from −916 nucleotides before the start of transcription. It therefore appears that the full length tobacco Nectarin I promoter is composed of an amalgam of several promoter elements that function to spatially and temporally regulate expression in the nectary gland.

Examinination of the Nectarin I promoter for cis-acting regulatory elements revealed little information about either the tissue or temporal specificity of this promoter. However, Ge *et al.* (2000) have recently described a nectary expressed gene from petunia. When we compared the tobacco Nectarin I promoter with the promoter of the petunia gene (GenBank, AX006361), we find that there is little identity between these two sequences. However we did identify a nonaanucleotide sequence (Table 2) that is located in both promoters. This sequence contains a *myb* core sequence and has been previously implicated in the regulation of floral genes by *Antirrhinum majus myb340* gene (Jackson *et al.*, 1991, Sablowski *et al.*, 1994). In the tobacco gene, this sequence is located at nucleotides −899 to −891 relative to the transcription start site. It is present in both the pRT461 and the pRT500 constructs. In the petunia gene, this sequence is located at nucleotide position 400 to 408 and is repeated a second time at nucleotides 1621 to 1629 of the promoter. Whether this sequence is indeed involved in the regulation of these nectary genes will require additional work, but we note that we have recently cloned a cDNA from tobacco nectaries that appears to be the tobacco homolog of the *Antirrhinum majus myb340* gene (Carter and Thornburg, unpublished).

It is also of interest to note that the expression of pRT500 occurs in the nectary and in the petals but does not occur in the floral tube. An examination of the petals and the floral tube does not reveal large differences between these two organs. Nevertheless, it is clear from these studies that these organs have the capacity to express different genes. It is also interesting to note that the opening of the petals and the maturation of the nectary leading to nectar secretion need to occur at the same time. Because of the unique expression pattern for the pRT500 construct, these studies may have uncovered one mechanism whereby the coordination of these two events may be controlled.

The faithful expression of the Nectarin I promoter in the nectary and at times when nectar is being actively secreted leads us to conclude that this promoter would be extremely useful to engineer modifications in plant nectars that could either affect insect visitation or could produce novel secreted biochemicals. Because nectar contains only a limited array of secreted proteins, novel secreted proteins could be readily isolated in a nearly pure form from plant nectars.

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