The Plant Cell, Vol. 8, 1137–1148, July 1996 © 1996 American Society of Plant Physiologists

Evolution of Floral Scent in *Clarkia*: Novel Patterns of *S*-Linalool Synthase Gene Expression in the *C. breweri* Flower

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Flowers of *Clarkia breweri*, an annual plant from the coastal range of California, emit a strong sweet scent of which *S*-linalool, an acyclic monoterpene, is a major component. Chromosomal, chemical, and morphological data, and the species' geographic distribution, suggest that *C. breweri* evolved from an extant nonscented species, *C. concinna*. A cDNA of *Lis*, the gene encoding *S*-linalool synthase, was isolated from *C. breweri*. We show that in *C. breweri*, *Lis* is highly expressed in cells of the transmitting tract of the stigma and style and in the epidermal cells of petals, as well as in stamens, whereas in the nonscented *C. concinna*, *Lis* is expressed only in the stigma and at a relatively low level. In both species, changes in protein levels parallel changes in mRNA levels, and changes in enzyme activity levels parallel changes in protein levels. The results indicate that in *C. breweri*, the expression of *Lis* has been upregulated and its range enlarged to include cells not expressing this gene in *C. concinna*. These results show how scent can evolve in a relatively simple way without the evolution of highly specialized "scent glands" and other specialized structures. *Lis* encodes a protein that is structurally related to the family of proteins termed terpene synthases. The protein encoded by *Lis* is the first member of this family found to catalyze the formation of an acyclic monoterpene.

INTRODUCTION

For many plants, species-specific floral scent is an important factor in attracting specific pollinators (Dobson, 1993). The scents of highly evolved flowers, such as orchids, are complex mixtures of volatiles (Knudsen and Tollsten, 1993) emitted from specialized "scent glands" (Stern et al., 1986). However, little is known about the initial steps in the evolution of floral scent. The ability to produce scent appears to be a relatively easily acquired trait. In the evening primrose family (Onagraceae), for example, moth-attracting scented species have evolved independently at least four times (MacSwain et al., 1973; Raven, 1979). Volatiles emitted by flowers encompass many classes of secondary metabolites, of which monoterpenoids constitute a commonly occurring class (Knudsen et al., 1993). However, because little has been known about enzymes and genes controlling scent production in any plant species, an understanding of the molecular mechanisms underlying scent production and the evolution of a scent could not be attempted until now.

We have recently begun to study the scent produced by flowers of *Clarkia breweri*, an annual plant native to the coastal range of California (Lewis, 1955). The genus *Clarkia*, which is subdivided into eight sections, is a member of the evening primrose family and contains 44 species. With the exception of the moth-pollinated *C. breweri* (section Eucharidium), all other species of the genus have essentially nonscented flowers that are pollinated mostly by bees (MacSwain et al., 1973). The flowers of *C. breweri*, a species believed to have evolved recently from the nonscented *C. concinna* (the only other member of section Eucharidium), emit a strong, sweet scent consisting of a relatively simple mixture of monoterpenoid and benzoid compounds (Raguso and Pichersky, 1995).

Of the terpenoids emitted by *C. breweri* flowers, a major constituent is *S*-linalool, an acyclic monoterpene alcohol. *S*-Linalool is produced in a one-step reaction from geranyl pyrophosphate (GPP), a ubiquitous intermediate in the biosynthesis of a variety of terpenoids, in a reaction catalyzed by the monomeric enzyme *S*-linalool synthase (LIS) (Pichersky et al., 1995). LIS activity is detected in *C. breweri* only in floral tissues. The four petals, which constitute 40% of the total mass of the flower, contain 50 to 70% of the total LIS activity (depending on the particular stage of development), whereas the upper pistil (i.e., stigma and style), which constitutes 10% of the total mass of the flower, contains as much as 50% of the total LIS activity in the flower (Pichersky et al., 1994). Some low levels of LIS activity are also detected in stamens, but no activity is found in ovaries or sepals (Pichersky et al., 1994). LIS activity in stigmata and other flower parts is first detected in late buds, and activity levels for the various floral organs peak during the first 2 days after the flower opens, with a corresponding peak in

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S-linalool emission (Pichersky et al., 1994). Interestingly, a low level of LIS activity (~3% of that found in *C. breweri* stigmata) was detected in the stigmata of *C. concinna* (Pichersky et al., 1994).

No specialized scent glands or similar structures have been found in *C. breweri* (Raguso and Pichersky, 1995). This is consistent with the observation that the scent is a newly evolved trait in this species, and specialized structures may not have had time to evolve. Whereas our work cited above showed a large increase in, and a wider distribution of, LIS activity in *C. breweri* flowers as compared with *C. concinna* flowers, these observations by themselves did not reveal whether such changes were brought about by changes in the level of *Lis* gene transcription or by changes at subsequent steps controlling gene expression. Nor could it be determined whether all or only some types of cells within a floral organ express the *Lis* gene. Here, we set out to determine the specific types of cells in the *C. breweri* flower that express the *Lis* gene and to identify the specific point(s) of control responsible for the high levels of *Lis* expression in this species. By comparing the results with those from *C. concinna*, we may begin to understand the initial steps in the evolution of scent in *Clarkia*.

RESULTS

Isolation and Characterization of *C. breweri Lis* cDNA Clones

We described previously the purification of LIS, a monomeric enzyme that catalyzes the conversion of GPP to the floral scent component *S*-linalool, from flowers of the annual plant species *C. breweri* (Pichersky et al., 1995). In this study, we determined the amino acid sequence of the first 32 residues from the N terminus as well as the sequence of two internal regions of 26 and 15 residues (Figure 1). We then employed the amino acid sequence of the N terminus to construct oligonucleotides to amplify a short segment (68 nucleotides) of the *Lis* coding region, using DNA from a *C. breweri* flower cDNA library as the target. The amplified fragment was used in turn to screen the same cDNA library. Among the clones that were initially identified, 15 were chosen for further analysis by restriction digests and nucleotide sequence determination.

All 15 clones were found to have the same nucleotide sequence within their overlapping regions. The nucleotide sequence of the longest cDNA, containing 2760 nucleotides, is presented in Figure 1. Primer extension experiments (data not shown) indicate that this clone is missing only 22 nucleotides from its 5′ end. Analysis of the nucleotide sequence around the first ATG codon, at positions 32 to 34, suggests that it is the initiating codon (Lütcke et al., 1987). The reading frame that begins with this methionine codon has 870 codons, and it ends with a TAA stop codon at positions 2642 to 2644 (Figure 1). The experimentally determined sequences of two

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                                                                AGAAAACCAAACCACCTTAAACAAGACAACC   31
                ATGCAGCTCATAACAAATTTCTCCTCATCATCATCAGAATTGCAGTTTCTTGTGGATAAG   91
              1 M  Q  L  I  T  N  F  S  S  S  S  S  E  L  Q  F  L  V  D  K
                GTTAAGAGAGAATCATTGTCTTCTTCATCATCTAATACTCAGAATTTGTTTCTCTCAACT  151
             21 V  K  R  E  S  L  S  S  S  S  S  N  T  Q  N  L  F  L  S  T
                TCACCTTATGACACTGCTTGGCTCGCCCTTATCCCTCATCCTCATCATCACCATCACCAT  211
             41 S  P  Y  D  T  A  W  L  A  L  I  P  H  P  H  H  H  H  H  H
                GGCCGACCCATGTTTGAAAAATGTCTGCAATGGATTCTCCATAACCAGACACCACAAGGT  271
             61 G  R  P  M  F  E  K  C  L  Q  W  I  L  H  N  Q  T  P  Q  G
                TTCTGGGCAGCAGCTGGTGACAATATTTCCGACACCGACGATGACGTCACCCTGGATTGT  331
             81 F  W  A  A  A  G  D  N  I  S  D  T  D  D  D  V  T  L  D  C
                CTTCTATCAACCTTGGCTTGCTTAGTTGCACTCAAAAGGTGGCAGCTTGCTCCCGACATG  391
            101 L  L  S  T  L  A  C  L  V  A  L  K  R  W  Q  L  A  P  D  M
                ATTCATAAAGGATTGGAATTTGTAAATAGAAACACAGAGAGACTTGTAATGAAGCAGAAG  451
            121 I  H  K  G  L  E  F  V  N  R  N  T  E  R  L  V  M  K  Q  K
                CCGAGCGACGTTCCTCGTTGGTTCACCATCATGTTCCCGGCGATGCTCGAGCTTGCCGGA  511
            141 P  S  D  V  P  R  W  F  T  I  M  F  P  A  M  L  E  L  A  G
                GCTTCCAGTCTCCGAGTCGATTTCAGCGAGAATCTTAACAGAATCTTGGTGGAACTATCT  571
            161 A  S  S  L  R  V  D  F  S  E  N  L  N  R  I  L  V  E  L  S
                CAAAATAGGGATGATATTCTCACAAGGGAGGAAGTTGATGAGAAGAAGCAATACTCACCA  631
            181 Q  N  R  D  D  I  L  T  R  E  E  V  D  E  K  K  Q  Y  S  P
                TTGCTACTATTTCTAGAAGCATTGCCTGCACAATCCTATGACAATGATGTTCTAAAGCAA  691
            201 L  L  L  F  L  E  A  L  P  A  Q  S  Y  D  N  D  V  L  K  Q
                ATTATAGACAAGAACTTGAGCAATGATGGTTCTTTATTGCAATCGCCTTCTGCTACAGCA  751
            221 I  I  D  K  N  L  S  N  D  G  S  L  L  Q  S  P  S  A  T  A
                AGAGCATACATGATAACAGGAAATACCAGATGCTTATCGTATCTACACTCTTTAACAAAT  811
            241 R  A  Y  M  I  T  G  N  T  R  C  L  S  Y  L  H  S  L  T  N
                AGCTGCTCTAATGGAGGAGTACCATCATTCTATCCTGTTGACGACGACCTCCATGATCTT  871
            261 S  C  S  N  G  G  V  P  S  F  Y  P  V  D  D  D  L  H  D  L
                GTCATGGTGAATCAACTGACAAGGTCGGGTTTGACTGAACATCTCATCCCGGAGATTGAC  931
            281 V  M  V  N  Q  L  T  R  S  G  L  T  E  H  L  I  P  E  I  D
                CACCTTCTACTCAAAGTTCAAAAGAACTACAAATACAAAAAGCATCACCAAAATCATTG  991
            301 H  L  L  L  K  V  Q  K  N  Y  K  Y  K  K  A  S  P  K  S  L
                TATAGCATTGCTGCGGAACTATACAGGGATTCATTAGCATTTGGTTGCTTCGAGTCAAT 1051
            321 Y  S  I  A  A  E  L  Y  R  D  S  L  A  F  W  L  L  R  V  N
                AATCACTGGGTATCACCATCAATTTTTTGTTGGTTTTAGATGACGACGAAATCCGTGAT 1111
            341 N  H  W  V  S  P  S  I  F  C  W  F  L  D  D  D  E  I  R  D
                CACATCGAAACAAACTACGAGGAATTTGCTGCCGTGCTTCTTAATGTGTATCGAGCTACC 1171
            361 H  I  E  T  N  Y  E  E  F  A  A  V  L  L  N  V  Y  R  A  T
                GATCTTATGTTCTCCGGCGAAGTCCAACTTGTCGAAGCAAGATCTTTCGCTACCAAGAAT 1231
            381 D  L  M  F  S  G  E  V  Q  L  V  E  A  R  S  F  A  T  K  N
                CTTGAGAAAATATTAGCAACAGGAAACATACATAAAACTAATGCAGATATCTCATCTAGT 1291
            401 L  E  K  I  L  A  T  G  N  I  H  K  T  N  A  D  I  S  S  S
                TTGCATAAGATGATCGAACACGAACTAAGAGTTCCTTGGACCGCAAGAATGGACCATGTT 1351
            421 L  H  K  M  I  E  H  E  L  R  V  P  W  T  A  R  M  D  H  V
                GAAAATCGAATTTGGATCGAAGAAATAGCTTCCAGTGCTTTATGGTTTGGAAAATCATCC 1411
            441 E  N  R  I  W  I  E  E  I  A  S  S  A  L  W  F  G  K  S  S
                TACCTTAGGTTATCTTGCTTTCACAAGATGAGTTTACAGCAACTCGCGGTGAAAAATTAT 1471
            461 Y  L  R  L  S  C  F  H  K  M  S  L  Q  Q  L  A  V  K  N  Y
                ACGCTTCGACAATTGGTTTACCGAGACGAGCTTGCGGAAGTTGAGAGGTGGTCTAAAGAA 1531
            481 T  L  R  Q  L  V  Y  R  D  E  L  A  E  V  E  R  W  S  K  E
                AGAGGGCTATGTGACATGGGATTTTGTAGAGAGAAAACCGGGTATTGTTACTACGCATTT 1591
            501 R  G  L  C  D  M  G  F  C  R  E  K  T  G  Y  C  Y  Y  A  F
                GCGGCAAGTACTTGTCTGCCGTGGAGTTCCGACGTGAGGCTGGTCCTGACCAAGGCGGCA 1651
            521 A  A  S  T  C  L  P  W  S  S  D  V  R  L  V  L  T  K  A  A
                GTTGTCATTACAGTGGCCGATGATTTCTTTGATGTCGAAGGATCTATGGTTGATCTCGAA 1711
            541 V  V  I  T  V  A  D  D  F  F  D  V  E  G  S  M  V  D  L  E
                AAATTAACGGATGCAGTTCGGAGGTGGGATCGCGGAAGGGTTAGGCAGCCACAGCAAGACA 1771
            561 K  L  T  D  A  V  R  R  W  D  A  E  G  L  G  S  H  S  K  T
                ATATTTGAAGCCCTGGATGATCTTGTAAATGAAGTTAGACTCAAGTGTTTCCAACAAAAT 1831
            581 I  F  E  A  L  D  D  L  V  N  E  V  R  L  K  C  F  Q  Q  N
                GGACAAGACATCAAAAACAATCTCCAACAATTATGGTATGAAACATTCCATTCATGGCTT 1891
            601 G  Q  D  I  K  N  N  L  Q  Q  L  W  Y  E  T  F  H  S  W  L
                ATGGAAGCTAAGTGGGGAAAGGGGTTAACAAGTAAACCATCTGTAGATGTGTATCTTGGA 1951
            621 M  E  A  K  W  G  K  G  L  T  S  K  P  S  V  D  V  Y  L  G
                AATGCAATGACATCCATAGCAGCTCACACCATGGTCCTTACAGCATCCTGTCTTCTAGGT 2011
            641 N  A  M  T  S  I  A  A  H  T  M  V  L  T  A  S  C  L  L  G
                CCCGGTTTCCCGGTTCACCAACTATGGTCGCAAAGGCGCCACCAGGACATTACATCCTTG 2071
            661 P  G  F  P  V  H  Q  L  W  S  Q  R  R  H  Q  D  I  T  S  L
                CTCATGGTCTTGACTCGCTTGCTAAATGACATTCAATCCTACTTGAAAGAAGAAGACGAA 2131
            681 L  M  V  L  T  R  L  L  N  D  I  Q  S  Y  L  K  E  E  D  E
                GGAAAAATAAACTATGTATGGATGTACATGATCGAGAACAATCAAGCGTCGATAGATGAC 2191
            701 G  K  I  N  Y  V  W  M  Y  M  I  E  N  N  Q  A  S  I  D  D
                TCGGTTCGACACGTCCAGACGATAATCAATGTAAAAAAGCAAGAATTCATCCAACGTGTT 2251
            721 S  V  R  H  V  Q  T  I  I  N  V  K  K  Q  E  F  I  Q  R  V
                CTATCGGATCAACATTGCAATCTCCCAAAGTCATTCAAGCAGCTCCATTTCTCCTGCCTC 2311
            741 L  S  D  Q  H  C  N  L  P  K  S  F  K  Q  L  H  F  S  C  L
                AAAGTATTCAACATGTTCTTCAACTCCTCCAACATTTTCGACACTGATACCGACCTTCTT 2371
            761 K  V  F  N  M  F  F  N  S  S  N  I  F  D  T  D  T  D  L  L
                CTTGACATTCACGAAGCTTTTGTTTCTCCACCACAAGTTCCCAAATTCAAACCCCACATC 2431
            781 L  D  I  H  E  A  F  V  S  P  P  Q  V  P  K  F  K  P  H  I
                AAGCCACCTCATCAGCTTCCAGCAACACTTCAGCCACCTCATCAGCCCCAACAAATAATG 2491
            801 K  P  P  H  Q  L  P  A  T  L  Q  P  P  H  Q  P  Q  Q  I  M
                GTCAATAAGAAGAAGGTGGAAATGGTTTACAAAAGCTATCATCATCCATTCAAGGTTTTC 2551
            821 V  N  K  K  K  V  E  M  V  Y  K  S  Y  H  H  P  F  K  V  F
                ACCTTGCAGAAGAAACAAAGTTCGGGACATGGTACAATGAATCCAAGGGCTAGTATCTTA 2611
            841 T  L  Q  K  K  Q  S  S  G  H  G  T  M  N  P  R  A  S  I  L
                GCAGGACCCAACATCAAACTATGTTTCAGTTAACGAATACACTACCTTGTTATTAGAAGA 2671
            861 A  G  P  N  I  K  L  C  F  S  *
                TGTCACCAGTTTCCAAACTCATCTGCTATGTATTTACATATCATGTGATAAGCAAAATTC 2731
                TCTAATAATCTATCCTTTTTTATGTCAAA                                2760
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Figure 1. Nucleotide and Deduced Amino Acid Sequences of *C. breweri Lis* cDNA.

The predicted amino acid sequence is shown directly below the nucleotide sequence. The asterisk indicates the stop codon. Experimentally determined amino acid sequences are underlined. The *Lis* EMBL accession number is U58314.

internal peptides of the purified LIS protein, as well as its N-terminal sequence, are found within this open reading frame (Figure 1), verifying its identity as a *Lis* cDNA. The results of DNA gel blots suggest that *Lis* is a single-copy gene in both *C. breweri* and *C. concinna* (data not shown).

The open reading frame of *Lis* contains eight additional amino acids at the N terminus compared with the N-terminal sequence of the purified protein, indicating either that LIS undergoes a proteolytic processing event in the cell or that cleavage of the protein occurs during the purification procedure. The calculated molecular mass of the precursor protein encoded by the *Lis* cDNA is 99.7 kD. The molecular weight of the native, monomeric LIS protein was previously determined by several methods to be in the range of 73 to 90 kD (Pichersky et al., 1995; L. Cseke and E. Pichersky, unpublished data). The discrepancy between the calculated and experimentally determined molecular mass is probably due partly to the eight residues missing from the N terminus and partly to the apparently tight packing of the native protein, which contains 16 cysteine residues. Evidence for internal disulfide bridges in the LIS protein has been described by Pichersky et al. (1995).

Comparisons of the deduced LIS amino acid sequence with sequences of other terpene synthases revealed a region of high sequence similarity centered around the conserved motif DDXXD (Figure 2A), which is also found in a related class of enzymes, the prenyl transferases, and is believed to be

A

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LIS   472  LQQLAVKNYT LRQLVYRDEL AEVERWSKER GL-QDMGFCR EKTGYCYYAF  520
LMS   283  VLELAILDLN IVQAQFQEEL KESFRWWRNT GFVEKLPFAR DRLVECYFWN  332
VES   238  LLRFAKLDYN LLQMLHKHEL SEVSRWWKDL DFVTTLPYAR DRAVECYFWT  287
EAS   233  LLRFAKLDFN LLQMLHKQEL AQVSRWWKDL DFVTTLPYAR DRVVECYFWA  282
CAS   279  LLEFAKLDYN RVQLLHQQEL CQFSKWWKDL NLASDIPYAR DRMAEIFFWA  328

LIS   521  AASTCLPWSS DVRLVLIKAA VVITVADDFF DVEGSMVDLE KLTDAVRRWD  570
LMS   333  TGIIEPRQHA SARIMMGKVN ALITVIDDIY DVYGTLEELE QFTDLIRRWD  382
VES   288  MGVYAEPQYS QARVMLAKTI AMISIVDDIF DAYGIVKELE VYTDAIQRWD  337
EAS   283  LGVYFEPQYS QARVMLVKTI SMISIVDDIF DAYGIVKELE AYTDAIQRWD  332
CAS   329  VAMYFEPDYA HTRMIIAKVV LLISLIDDTI DAYATMEETH IFAEAVARWD  378
                                 DDXX_D
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B

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LIS    39  STSPYDTAWL ALIPHPHHHH HHGRPMFEKC LQWILHNQIP QGFWAAAGDN   88
GA1   114  TISAYDTAWV ALID-----A GDKTPAFPSA VKWIAENQLS DGSWGDAYLF  158
An1   110  SASAYDTAWV AMVP-KVGGD GGAQPQFPAT VRWIVDHQLP DGSWGDSALF  158

LIS    89  ISDTDDDVTL DCLLSTLACL VALKRWQLAP  118
GA1   159  S-------YH DRLINTLACV VALRSWNLFP  181
An1   159  S-------AY DRMINTLACV VALTKWSLEP  181

LIS   226  LSNDGSLLQS PSATARAYMI TGNTRCLSYL HSLTNSCSNG GVPSFYPVDD  275
GA1   280  QSQDGSFLFS PSSTAFAFMQ TRDSNCLEYL -RNAVKRFNG GVPNVFPVDL  328
An1   281  QSQDGSFLFS PSATAYALMQ TGDKKCFEYI -DRIVKKFNG GVPNVYPVDL  329
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Figure 2. Comparison of the Predicted Amino Acid Sequence of *C. breweri* LIS Protein to Related Proteins.

(A) Alignment of sequences of terpene synthases around the conserved DDXXD motif. The sequence of *C. breweri* LIS between residues 472 and 570 is aligned with the spearmint 4S-limonene synthase (LMS) sequence between residues 283 to 382 (Colby et al., 1993; EMBL accession number L13459), *H. muticus* vetispiradiene synthase (VES) sequence between residues 238 to 337 (Back and Chappell, 1995; EMBL accession number U20187), tobacco 5-epiaristolochene synthase (EAS) sequence between residues 233 to 332 (Facchini and Chappell, 1992; EMBL accession number L04680), and *R. communis* casbene synthase (CAS) sequence between residues 279 to 378 (Mau and West, 1994; EMBL accession number L32134). Identical residues shared between the LIS sequence and any other protein are shown in black boxes with white lettering.
(B) Segments of *C. breweri* LIS from residues 39 to 118 and 226 to 285 aligned with Arabidopsis *ent*-kaurene synthase A (GA1) segments 114 to 181 and 280 to 328 (Sun and Kamiya, 1994; EMBL accession number U11034) and maize *ent*-kaurene synthase A (An1) segments 110 to 181 and 281 to 329 (Bensen et al., 1995; EMBL accession number L37750).

involved in the binding of the divalent metal cofactor (Tarshis et al., 1994). The overall sequence identity of this region in the *C. breweri* LIS with the spearmint 4*S*-limonene synthase is 31%; with *Hyoscyamus muticus* vetispiradiene synthase, it is 34%; with tobacco 5-epiaristolochene synthase, it is 33%; and with *Ricinus communis* casbene synthase, it is 27%. When conservative substitutions are considered, the overall sequence similarity of LIS with any of these terpene synthases is ~60%. LIS does not share significant sequence similarity with known prenyl transferase sequences.

ent-Kaurene synthase A, an enzyme in the gibberellin pathway that catalyzes the cyclization of a diterpene without the removal of the pyrophosphate group, shows some similarity with other terpene synthases but lacks the DDXXD motif (Sun and Kamiya, 1994; Bensen et al., 1995). LIS also displays some sequence identity with *ent*-kaurene synthase A (Figure 2B). The two segments with the highest similarity, residues 39 to 118 and 226 to 275 of LIS, show 41 and 51% identity to Arabidopsis *ent*-kaurene synthase A residues 114 to 181 and 280 to 328, respectively, and 39 and 53% to maize *ent*-kaurene synthase A residues 110 to 181 and 281 to 329, respectively. Incidentally, the two *ent*-kaurene A synthases are the only available examples of two sequences of the same terpene synthase from two different species, and in the segments compared above, they show 58 and 71% sequence identity, respectively, with each other.

Tissue-Specific Expression of *Lis*

To determine tissue specificity of *Lis* expression, total RNA was isolated from *C. breweri* leaves and floral tissues (sepals, petals, stigma, style, and stamens) of 1-day-old flowers and used in RNA gel blot hybridizations (Figure 3A). *Lis* mRNA was detected in all of these floral tissues, except in the tissue of sepals, but the level of expression was different. The length of the message was estimated to be 3.1 kb, suggesting that the characterized *Lis* cDNA clone (Figure 1), which does not contain a poly(A) tail, is probably also missing 150 to 250 nucleotides from the 3′ nontranslated region. (One explanation of this observation is that the cDNA library was made using random oligonucleotides as primers for first-strand DNA synthesis.) *Lis* mRNA transcript accumulated to high levels in stigmata and styles. It was also present in petals and stamens but at lower levels. No detectable signal was found in leaves. The levels of 18S rRNA served as an internal control for the quantity of total RNA loaded in each lane (Figures 3 and 4).

We also examined the expression of *Lis* in 1-day-old flowers of *C. concinna*, a close relative of *C. breweri* that does not emit a detectable scent but does contain low levels of LIS enzymatic activity in the stigma (Pichersky et al., 1994). A weak hybridization signal from a 3.1-kb mRNA was detected by RNA gel blots only in the stigma (Figure 3B), and no signals were detected in petals, styles, stamens, or sepals after a 3-day exposure of the autoradiogram.

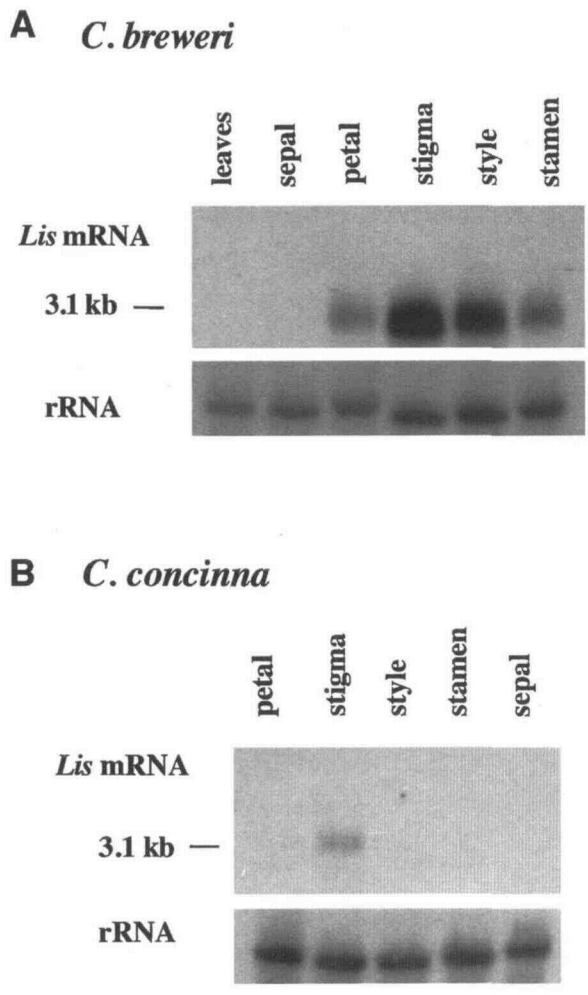

Figure 3. Tissue Specificity of *Lis* Gene Expression.

(A) RNA gel blot of total RNA isolated from leaves, sepals, petals, stigmata, styles, and stamens of 1-day-old open flowers of *C. breweri*. The top panel represents the results of hybridization with a *Lis* probe. The length of the *Lis* mRNA was estimated at 3.1 kb by using RNA molecular markers run in an adjacent lane. The blots were rehybridized with the 18S rDNA (bottom panel) for loading control.

(B) RNA gel blot of total RNA isolated from petals, stigmata, styles, stamens, and sepals of 1-day-old open flowers of *C. concinna*. The top panel represents results from hybridization with a *Lis* probe; the bottom panel represents results from probing with an 18S rDNA probe. mRNA size determination was done as described in **(A)**.

Developmental Modulation of *Lis* Expression

We next analyzed the temporal and spatial accumulation patterns of *Lis* mRNA in *C. breweri* floral organs. Total RNA was isolated from petals, stigmata, styles, and stamens of flowers at different times after anthesis (1 to 4 days) and also from buds at stage 5 (1 day before opening) and stage 4 (Pichersky

et al., 1994). Some *Lis* mRNA was found in pistil (stigma and style) tissue at stage 4 buds (several days before opening), with trace amounts found in petals as well (Figures 4A and 4B). Expression of petal *Lis* mRNA peaked in buds at stage 5. At this stage, 1 day before anthesis, *Lis* mRNA levels were approximately three and five times higher in petals than in pistil and stamens, respectively (Figure 4B).

In open flowers (days 1 to 4 postanthesis), the highest level of *Lis* expression was found in the stigma with a peak on day 1. During the lifespan of the flower, the *Lis* gene is also highly expressed in the style. A relatively low transcript level was found in stamens. The maximal level of *Lis* expression in the style was stable during days 1 and 2, whereas in the other floral tissues *Lis* transcription dropped off significantly on the second day. For example, in contrast to the high level of *Lis* mRNA present in petals of stage 5 buds, the amount of *Lis* transcripts was significantly decreased in day 1 petals and was approximately three times less than that found in petals from buds. Less precipitous declines from the peak on day 1 were observed for the stigmata and stamens. However, despite the gradual decline in the level of *Lis* mRNA in the stigma, this level was still relatively high on the fourth day, compared with peak levels of all other organs, except petals.

In Situ Localization of *Lis* Transcripts in Floral Tissues

The spatial distribution of *Lis* transcripts was examined in *C. breweri* stage 5 buds and in pistils and petals of day 1 flowers by in situ hybridization using *Lis* sense and antisense RNA probes. In cross-sections of flower buds, it can be seen that *Lis* mRNA transcripts are concentrated mainly in the secretory

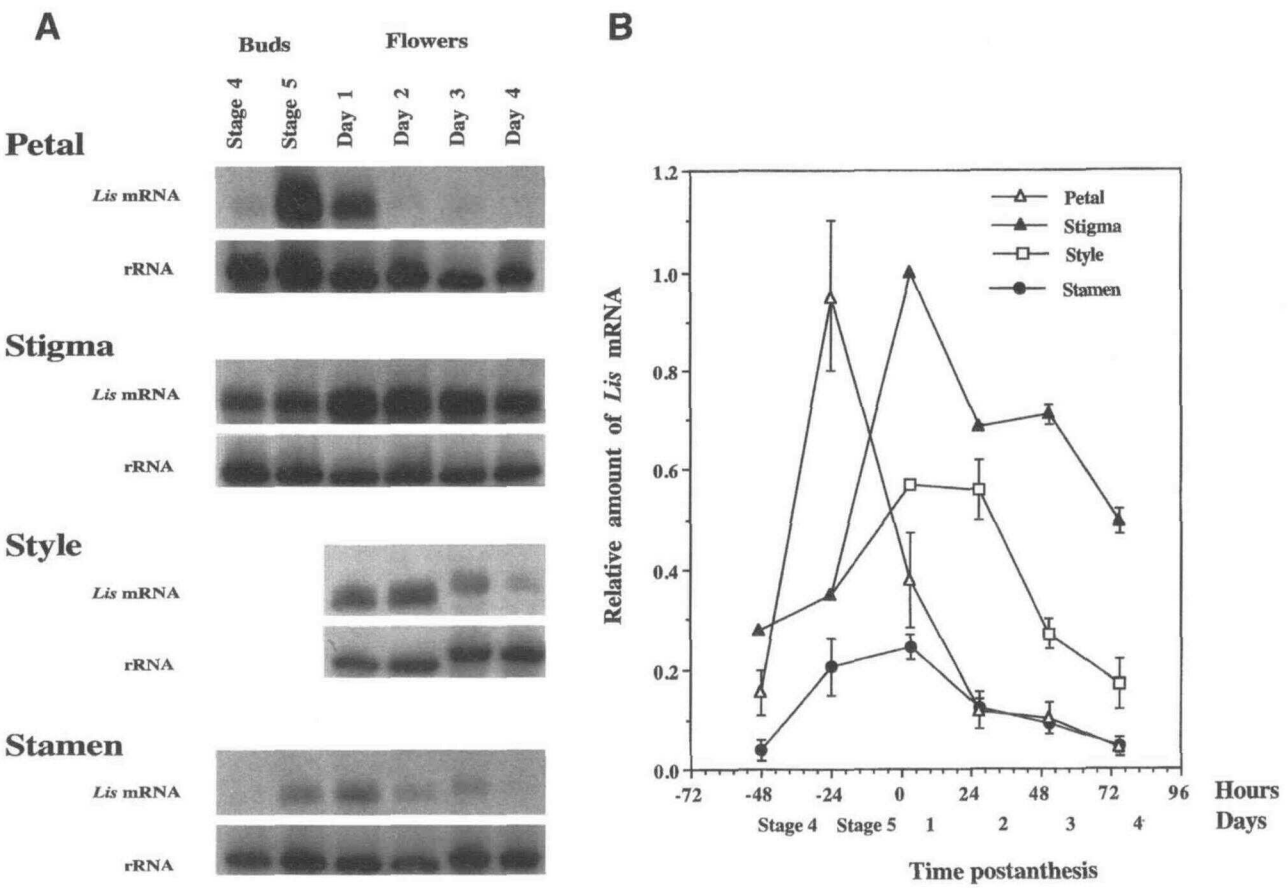

Figure 4. Developmental Modulation of *Lis* Expression in *C. breweri*.

(A) Autoradiography of RNA gel blots probed with *Lis* and reprobed with 18S rRNA. RNA was extracted from petals, stigmata, styles, and stamens of buds from stages 4 and 5 and of flowers open from 1 to 4 days.

(B) Relative *Lis* transcript levels in flower tissues as obtained by scanning of RNA gel blots, corrected for variation in total amount of RNA, using the hybridization results with the 18S rDNA probe for calibration. The data were derived from at least three independent experiments. Standard error values are indicated as vertical bars.

zone of the four-lobed stigma and also in epidermal layers of petals (Figures 5A, 5C, 5E, 5F, and 5H). Longitudinal sections through the upper region of the stigma of day 1 opened flowers are shown in Figures 6A to 6C. Strong *Lis* expression could be seen in the secretory zone of the stigma, located between the papillate epidermis and parenchyma cells (Figures 6A and 6C). The secretory zone converges into the style as a central region of transmitting tissue (Figures 6D to 6F). Within the style, *Lis* is specifically expressed in the transmitting tissue but not in the vascular system or stylar epidermis (Figures 6G and 6I). In day 1 petals, *Lis* transcripts were observed mainly in epidermal cells, similar to that of flower buds (Figures 5F, 5H,

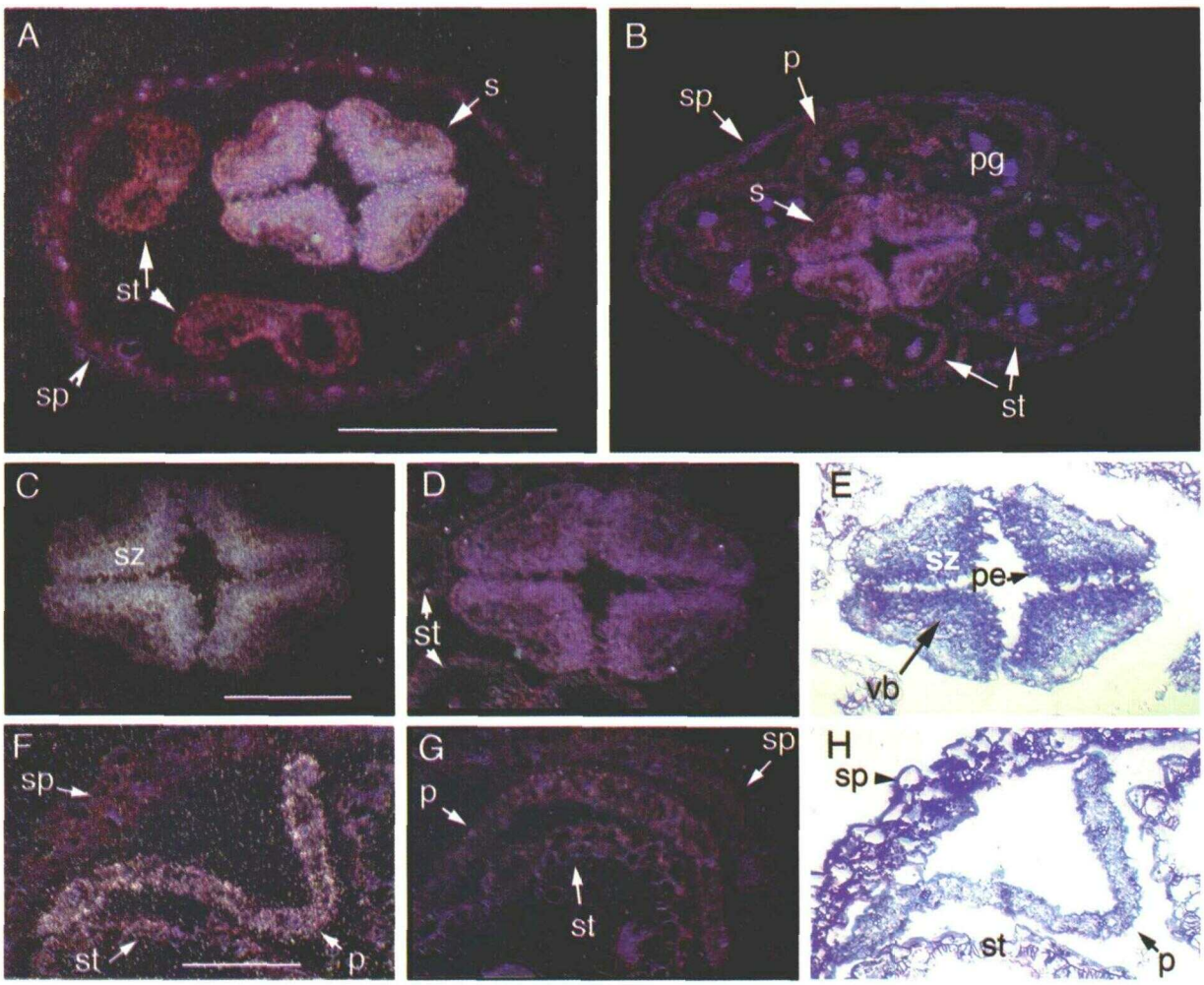

Figure 5. In Situ mRNA Localization of *Lis* Transcripts in *C. breweri* Flower Buds 1 Day before Anthesis (Opening of Flower).

Flower bud cross-sections were hybridized with the *Lis* antisense probe in **(A)**, **(C)**, **(E)**, **(F)**, and **(H)** and the sense probe in **(B)**, **(D)**, and **(G)**. Dark-field microscopy was used in **(A)** to **(D)**, **(F)**, and **(G)**, in which hybridization signals are visible as white dots. Bright-field microscopy of the sections in **(C)** and **(F)** are shown in **(E)** and **(H)**, respectively. The dye used in staining these sections is toluidine blue.

(A) and **(B)** *Cross-sections through a flower bud 1 day before flower opening.* Bar = 1 mm.

(C) to **(E)** Cross-sections through the stigma in the flower bud. Bar = 50 μm.

(F) to **(H)** Cross-sections through the petal in the flower bud. Bar = 25 μm.

p, petal; pe, papillate epidermis; pg, pollen grain; s, stigma; sp, sepal; st, stamen; sz, secretory zone; vb, vascular bundle.

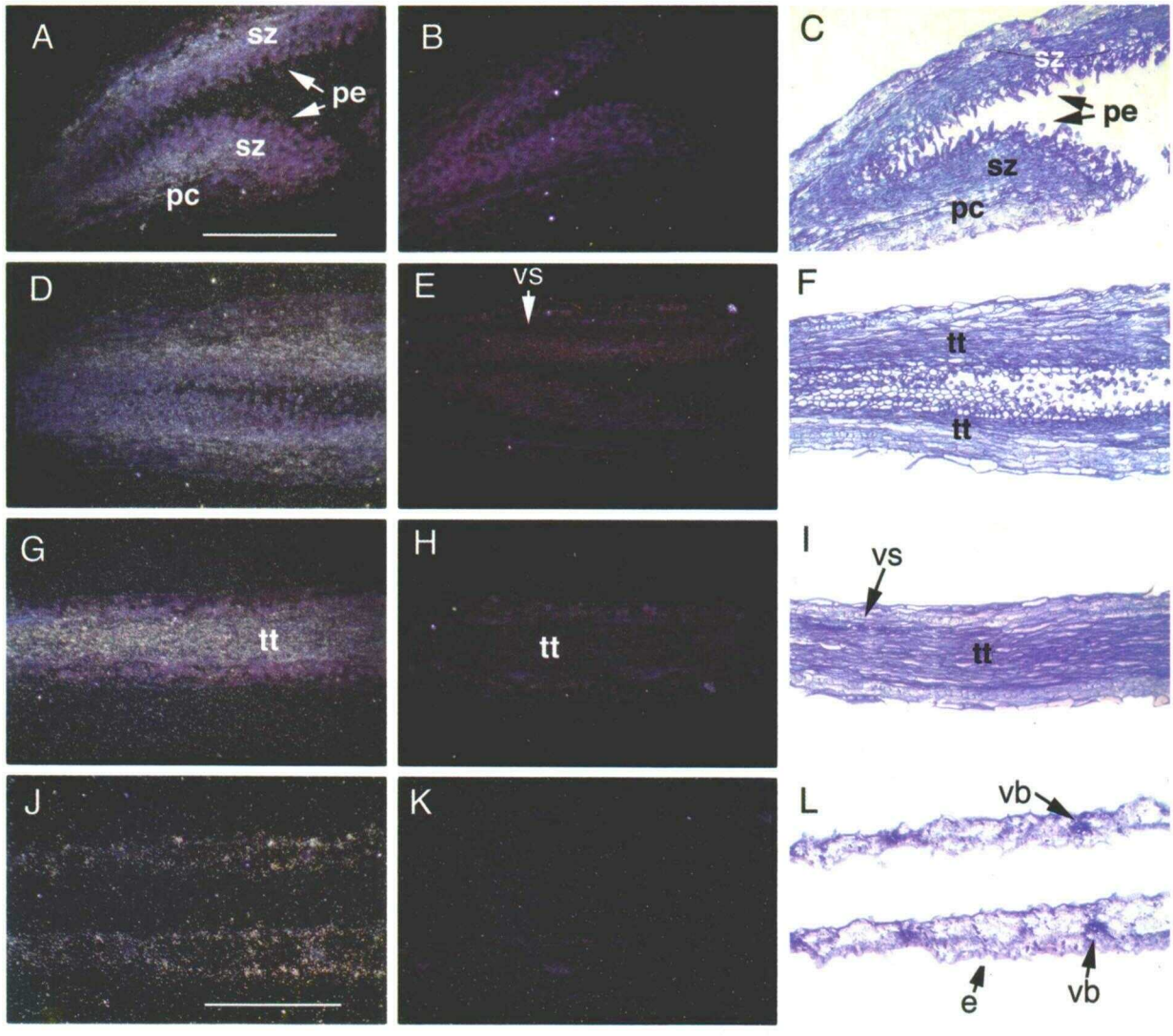

Figure 6. In Situ Hybridization of *Lis* mRNA Transcripts in Stigma, Style, and Petals of 1-Day-Old *C. breweri* Flowers.

Longitudinal sections of flower tissues were hybridized with the *Lis* antisense probe and are shown in **(A)**, **(C)**, **(D)**, **(F)**, **(G)**, **(I)**, **(J)**, and **(L)** and with the sense probe in **(B)**, **(E)**, **(H)**, and **(K)**. Dark-field microscopy was used in **(A)**, **(B)**, **(D)**, **(E)**, **(G)**, **(H)**, **(J)**, and **(K)**, and hybridization signals are visible as white dots. Bright-field microscopy of same sections shown in **(A)**, **(D)**, **(G)**, and **(J)** are shown in **(C)**, **(F)**, **(I)**, and **(L)**, respectively. The dye used in staining these sections is toluidine blue.

(A) to **(C)** Longitudinal sections through the upper part of the stigma. Bar in **(A)** = 50 μm for **(A)** to **(C)**.

(D) to **(F)** Longitudinal sections through two lobes of the stigma at the place of their separation from the style. Same magnification as in **(A)**.

(G) to **(I)** Longitudinal sections through the style. Same magnification as in **(A)**.

(J) to **(L)** Longitudinal sections through the petals. Bar in **(J)** = 25 μm for **(J)** to **(L)**.

e, epidermis; pc, parenchyma cells; pe, papillate epidermis; sz, secretory zone; tt, transmitting tissue; vb, vascular bundle; vs, vascular system.

6J, and 6L). No hybridization was detected when sense *Lis* RNA was used as a probe (Figures 5B, 5D, and 5G, and 6B, 6E, 6H, and 6K).

Developmental Modulations in the Levels of LIS Protein in Floral Tissue

The levels of LIS protein in *C. breweri* floral tissues over the developmental period from bud stage 4 to day 5 postanthesis were quantitatively determined by the chemoluminescence protein gel blotting technique. The polyclonal anti-LIS antibodies, prepared against the C-terminal third of the LIS protein expressed in *Escherichia coli*, recognized two closely migrating proteins with an apparent molecular mass of 84 kD in crude floral extracts separated by SDS-PAGE, and no other protein. The top protein band has been identified by N-terminal sequencing as LIS (Pichersky et al., 1995). The lower band, which is a minor component in most preparations (Figure 7A), may be a degradation product, an isozyme, or a different conformer of LIS. Under some conditions (e.g., the presence of urea and DTT in SDS-PAGE), only a single band in protein gel blots was observed (data not shown).

The protein blots (Figure 7A) and the measurements of the protein concentrations taken from these blots (Figure 7B) revealed that stigma and style tissues have the highest levels of LIS protein per gram fresh weight of tissue; this is consistent with previous observations that these tissues have the highest level of LIS enzymatic activity per gram fresh weight of tissue (Pichersky et al., 1994). The LIS protein levels in these tissues increase during bud development, peak at day 2 of anthesis, and remain relatively high for several days. Petals and filaments have lower levels of LIS per gram fresh weight of tissue, and in these tissues, the protein levels peak on day 1 of anthesis. Anthers exhibit relatively low levels of LIS protein, with a peak 1 day before anthesis. No LIS protein was detected in sepals and leaves (data not shown).

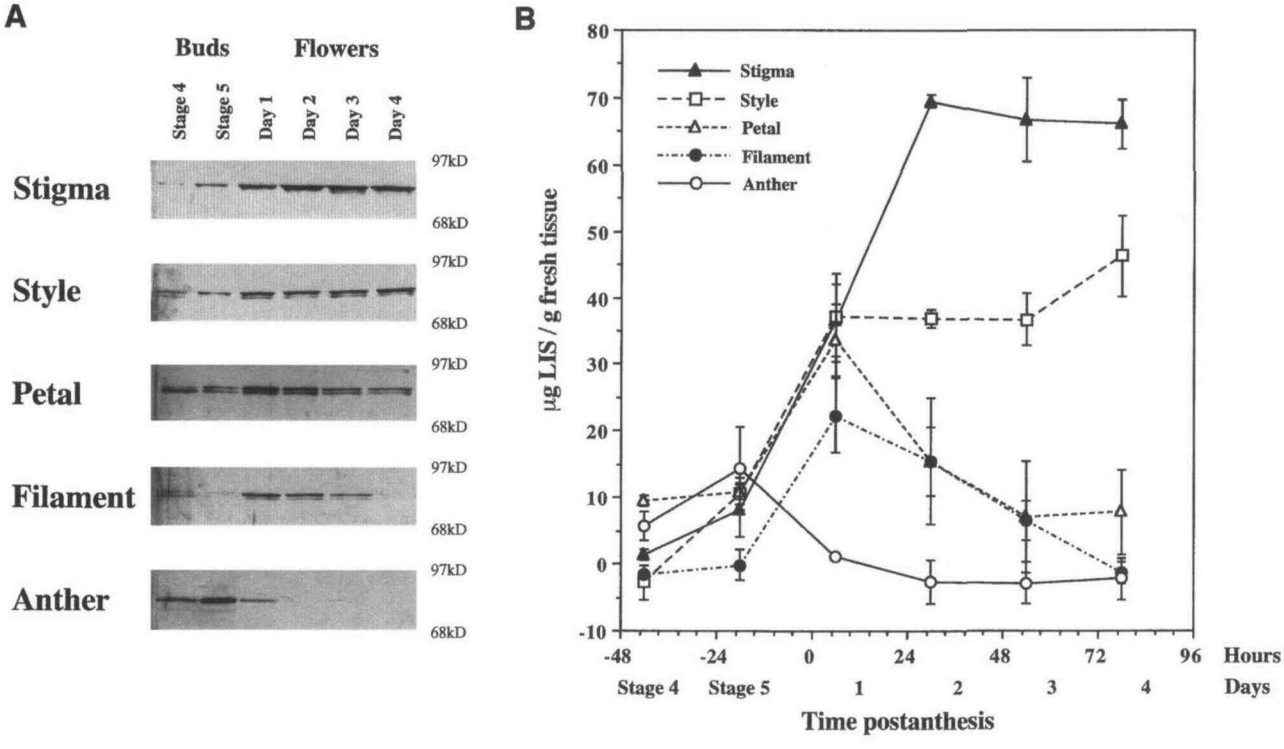

Figure 7. Protein Gel Blot Analysis of LIS Protein in Stigma, Style, Petals, Filament, and Anther during Development of Floral Organs.

(A) Representative protein gel blots of floral organs from bud stage 4 through day 4 of development showing the 84-kD protein recognized by anti-LIS antibodies. Each lane of each gel represents extracts obtained from the floral organs of 15 to 25 flowers. Numbers at right indicate the location of protein markers of known molecular mass in kilodaltons.

(B) LIS protein quantitation obtained by scanning the protein gel blots. Results are in µg LIS/g fresh tissue for each day of the developmental period. Error bars were calculated from two experiments.

DISCUSSION

Linalool Synthase Is a Member of the Terpene Synthase Protein Family

All terpene synthases bind a terpene precursor that contains a pyrophosphate group, whether it be GPP (monoterpene synthases; e.g., limonene synthase), Farnesyl pyrophosphate (sesquiterpene synthases; e.g., vetispiradiene synthase and 5-epiaristolochene synthase), or geranylgeranyl pyrophosphate (diterpene synthases; e.g., casbene synthase). With the exception of LIS, which catalyzes the synthesis of the acyclic monoterpene S-linalool, all terpene synthases whose sequence is currently known catalyze a reaction that results in a cyclic product. *ent*-Kaurene synthase A is another exception in this group because although it produces a cyclic product, copalyl pyrophosphate, this product still contains the pyrophosphate group, whereas all other products resulting from the catalysis by this group of enzymes—*S*-linalool, limonene, 5-epiaristolochene, vetispiradiene, and casbene—have lost the pyrophosphate group.

Previous investigations have shown that the sequence similarity between different terpene cyclases is not especially high (ranging from 31 to 42%). However, a region that contains the sequence DDXXD has been noted before (Mau and West, 1994; Chappell, 1995; McGarvey and Croteau, 1995) to be more highly conserved in all plant terpene synthases, with the exception of *ent*-kaurene synthase A. It is difficult to assess the overall sequence similarity between *C. breweri* LIS and the terpene cyclases because LIS is a much larger protein (870 residues compared with 550 to 600 residues). Our data show that although the amino acid sequence of LIS has low (but statistically significant) overall sequence identity with terpene synthases, several regions show higher levels of identity. In particular, a region of ~100 residues that contains the motif DDXXD shows 27 to 34% sequence identity with the corresponding region in other terpene synthases, and when conservative substitutions are equated, the overall sequence similarity in this region is ~60% (Figure 2A). Because this region is not conserved in *ent*-kaurene synthase A, perhaps its significance lies not in the cyclization step but rather in the interaction with and the removal of the pyrophosphate group, possibly mediated by the divalent metal cofactor which the DDXXD motif has been hypothesized to bind (Tarshis et al., 1994; Chappell, 1995; McGarvey and Croteau, 1995).

Although *ent*-kaurene synthase A lacks the DDXXD motif and the rest of the conserved region around it, two other segments in this protein show high sequence similarity with LIS (Figure 2B). Although the significance of these two segments is not known, they are located close to the N terminus, a region in both LIS and *ent*-kaurene synthase A that shows no sequence similarity to other terpene cylcases. *ent*-Kaurene synthase A is, like LIS, a larger protein (*ent*-kaurene A has 802 residues), and the additional amino acid sequence in these two enzymes, compared with other terpene synthases, appears to have been added to the N-terminal region. In summary, the LIS protein sequence appears to be a composite of sequences found in at least two types of terpene synthases. A more precise delineation of its evolution must await the characterization of additional sequences of terpene synthases.

Emission of *S*-Linalool from *C. breweri* Flowers Is Regulated at the Nucleic Acid Level

C. breweri flowers emit a strong scent, of which *S*-linalool and one of its oxides are major components (Raguso and Pichersky, 1995). We have shown previously that LIS activity is found in several parts of the *C. breweri* flower, whereas in *C. concinna*, a scentless close relative from which *C. breweri* arose (Raguso and Pichersky, 1995), low levels of LIS activity could only be found in the stigma (3% of that found in the stigma of *C. breweri*; Pichersky et al., 1994). The LIS activity levels determined by Pichersky et al. (1994) show strong positive correlation with the level of LIS protein in a given tissue (Figure 7), suggesting that differences in LIS activity in different tissues and at different stages of development are due primarily to changes in amounts of LIS protein and not to post-translational modification.

The data presented in this report also show that the level of LIS protein is tightly correlated with the steady state levels of *Lis* mRNA in *C. breweri* (Figures 4 and 7). This is also true in *C. concinna*, where the level of *Lis* mRNA in the stigma is very low (Figure 3B) and the amount of LIS protein is so low that it cannot be detected by the protein gel blotting technique (data not shown). In addition, our data show that there is a lag time of ~1 day between the peak levels of mRNA and the peak levels of LIS protein in petal and stigma tissues but not in styles and anthers.

Novel Expression Patterns of *Lis* in Floral Cells of *C. breweri*

Both *C. concinna* and *C. breweri* show *Lis* expression in the stigma, suggesting that this is an ancestral condition. It has been shown previously (Pichersky et al., 1994; Raguso and Pichersky, 1995) that the expression of LIS in the stigma of *C. breweri* leads to the emission of linalool oxides, not *S*-linalool. Linalool oxides are produced from *S*-linalool via an additional enzymatic step(s), and they also serve as pollinator attractants in *C. breweri* (Raguso and Pichersky, 1995; Raguso et al., 1996). However, in *C. concinna* stigmata, the levels of the *S*-linalool and linalool oxides produced are very low, and they do not seem to serve to attract pollinators (Raguso et al., 1995). Thus, the function of *Lis* expression in the stigma of *C. concinna* is still obscured.

The most significant observation concerning the expression of *Lis* in *C. breweri* is that in addition to being upregulated significantly in the stigma compared with *C. concinna*, *Lis* is also expressed in other types of floral tissues such as styles, filaments, and petals. Up to 70% of the total LIS activity in the

C. breweri flower is found in the petals, which are the major source of emitted *S*-linalool (Pichersky et al., 1994; Raguso and Pichersky, 1995). The in situ RNA hybridization experiments (Figures 5 and 6) show that *Lis* is expressed in petals preferentially in epidermal cells, leading to the production of water-insoluble *S*-linalool on the surface of the petals, a position from which it can most easily escape into the atmosphere. Thus, it appears that *C. breweri* has evolved its ability to emit large amounts of *S*-linalool simply by highly expressing *Lis* in the epidermal cells of the petals, without the concomitant development of specialized "scent glands," as has been found in some other plants such as orchids (Stern et al., 1986). Because the petals of *C. breweri* emit additional scent volatiles (benzyl acetate is another major component), it would be of interest to examine whether the mechanisms responsible for their production and emission have evolved in ways similar to those governing the production and emission of *S*-linalool. It would also be interesting to determine whether *C. breweri* has evolved concomitantly a reward system, such as producing nectar of appropriate composition and suitable for the moth pollinator.

METHODS

Protein Sequencing

We previously reported the purification of linalool synthase from stigmata of *Clarkia breweri* flowers (Pichersky et al., 1995). The purified *S*-linalool synthase protein was subjected to N-terminal sequencing in a protein sequencer (model 477; Applied Biosystems, Foster City, CA). A sequence of 32 residues was determined in this way (Figure 1). In addition, the whole protein was cleaved with cyanogen bromide, the digestion products were subjected to SDS-PAGE, and two additional peptides were isolated and sequenced (Figure 1) by using standard protocols.

cDNA Library

Total RNA was isolated from petals and stigmata of 1-day-old *C. breweri* flowers and from flower buds 1 day before opening (stage 5 buds; Pichersky et al., 1994) by using the protocol described by Lewinsohn et al. (1994). A cDNA library was constructed in the phage vector λ-ZapII by using poly(A)$^+$ mRNA (Stratagene), according to company protocols. Both poly(T) and random primers were used in first-strand DNA synthesis. The titer of the unamplified library was 1.9×10^6.

Isolation and Characterization of cDNA Clones

Two oligonucleotides designed to match the region encoding the N-terminal peptide were synthesized. These oligonucleotides were (1) the sense 23-mer oligonucleotide for the amino acid sequence QFLVDKVK (positions 7 to 14 from the N terminus) CA(AG)TT(TC)-(TC)C(ACGT)GT(ACGT)GA(CT)AA(AG)GT(ACGT)AA and (2) the antisense 17-mer for amino acid sequence NTQNLF (positions 24 to 29 from the N terminus) AA(ACGT)G(AG)(AG)TT(CT)TG(ACGT)GT(AG)TT. Polymerase chain reaction was performed on DNA extracted from the cDNA library by using these two oligonucleotides in a previously described protocol (Schwartz et al., 1991). The anticipated 68-nucleotide fragment was obtained and cloned into the vector pCRII (Invitrogen, San Diego, CA). The complete sequence of the 68-bp fragment was determined to ascertain that the rest of its sequence indeed encoded the experimentally determined amino acid sequence from position 15 to 23. After obtaining a positive result, the cloned 68-bp fragment was excised from the vector and used as a probe to screen the cDNA library. Fifteen cDNA clones were isolated, and their 5′ sequence was determined. All proved to have identical sequences within their overlapping regions. The sequence of both strands of the longest clone was determined by the dideoxy chain termination method (Sanger et al., 1977).

RNA Isolation and RNA Gel Blot Analysis

Total RNA was isolated from 0.1 g of frozen plant tissues by using the RNeasy Plant Total RNA Kit (Qiagen Inc., Chatsworth, CA). The amount of RNA was determined by using a spectrophotometer. RNA samples (2 μg per lane) were size fractionated by electrophoresis under denaturing conditions in vertical urea–agarose gels (Locker, 1979) at 4°C for 5 hr at 20 W and transferred to Hybond N+ membranes (Amersham). A 2.2-kb EcoRI *Lis* cDNA fragment containing the 5′ end of the gene was used as a probe, and hybridizations were performed in 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, 0.1 mM EDTA), 50% formamide, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), and 0.5% SDS at 37°C for at least 18 hr. Membranes were washed once at 37°C with 5 × SSPE, 0.5% SDS, and twice with 2 × SSPE at 65°C before being exposed to x-ray film. mRNA transcripts were quantified by using a molecular imager system (model GS-363; Bio-Rad). *Lis* mRNA transcript levels were normalized to 18S rRNA levels to overcome error in RNA quantitation by using spectrophotometry. In addition, all gels contained a standard RNA sample from stigma tissue for equalizing signals among gels.

In Situ Hybridization

Preparation of tissues and hybridization conditions were the same as those described by Drews et al. (1991), with modifications used by Sakai et al. (1995). The *Lis* ^{35}S-labeled sense and antisense mRNA probes were synthesized with T3 and T7 RNA polymerase from an EcoRV- or BamHI-linearized pBluescript SK+ vector containing 450 nucleotides of the 3′ end of *Lis* cDNA. Slides were coated with emulsion (NTB-2; Eastman Kodak) and exposed for 2 to 4 weeks at 4°C.

Protein Gel Blots

Crude extracts of *C. breweri* floral organ tissues (stigma, style, petal, filament, anther, and sepal) were made by macerating each tissue from 15 to 25 individual flowers in a mircocentrifuge tube containing extraction buffer (50 mM Tris, pH 6.9, 5 mM sodium bisulfite, 10% glycerol, 1% insoluble PVPP, 2% SDS) in a ratio of 3:1 buffer per tissue fresh weight for 1 min by using a hand-held homogenizer. Extracts were made from the last two stages of bud development (stages 4 and 5) and four stages of open flower development (day 1 through day 4). All extracts

were spun for 10 min in a microcentrifuge at 13,000 rpm to remove cell debris and PVPP. The supernatants were prepared for SDS-PAGE by adding an equal volume of loading buffer (125 mM Tris, pH 6.8, 0.05% bromophenol blue, 20% glycerol, 10% β-mercaptoethanol, 4% SDS) and heating for 5 min at 95°C.

Samples (20 μL of each extract) were loaded on 10% SDS–polyacrylamide mini-gels along with samples of four known concentrations of purified native LIS. Gels were run at 200 V for 45 min. Transfer of proteins to nitrocellulose membranes was performed at 100 V for 1.5 hr in 48 mM Tris, 40 mM glycine, 0.04% SDS, and 20% methanol at 4°C in a Bio-Rad Mini Trans-Blot Cell.

Filters were blocked for 1 hr with PBS solution (pH 7.4) containing 0.5% Tween-20 (PBST) and 5% Carnation nonfat dry milk. Blocked filters were washed twice in PBST for 5 min to remove the milk. LIS protein bands were visualized by using Western Blot Chemiluminescence Reagent (Du Pont–New England Nuclear), according to the manufacturer's protocols except for the following modifications. Primary anti-LIS antibody (diluted 1:1540) and secondary goat anti–rabbit IgG horseradish peroxidase conjugate (diluted 1:30,000) were prepared in PBST instead of PBST plus 1% BSA. Incubations with primary and secondary antibody were performed for 1 hr at room temperature.

Bands were visualized on Kodak X-OMAT AR film. Quantitation was performed by using a molecular imager system (model GS-363; Bio-Rad) to scan images from 5-hr exposures onto an imaging screen (model CH GS-250; Bio-Rad) into a computer file using the Molecular Analyst program (Bio-Rad). The four standards from each protein gel blot for each tissue were used to produce standard curves for each given blot. These standard curves along with the initial data from tissue collection were then used to calculate concentration of LIS protein (in micrograms per gram fresh weight of tissue).

ACKNOWLEDGMENTS

This work was supported by National Science Foundation Grant No. IBN-9417582. L.C. is a recipient of a National Institutes of Health (NIH) training grant fellowship in biotechnology (No. 5T32GM08353). V.M.B. is a recipient of an NIH training grant fellowship in genetics (No. 5T32GM07544). We thank Dr. R.A. Raguso for help in growing the plants.

Received March 29, 1996; accepted May 21, 1996.

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