Journal of Plant Biology, March 2000, 43(2) : 107-113 **SHORT COMMUNICATION**

Analyses of Expressed Sequence Tags of Anther and Anther-Specific cDNA Clones in *Nicotiana tabacum*

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Expressed sequence tag (EST) analysis of tobacco (*Nicotiana tabacum*) anthers was performed for 200 cDNA clones randomly chosen from a cDNA library representing transcripts of developing tobacco anthers. Only 21 clones showed significant homology to the nucleotide sequences in databases. Most of these belonged to the housekeeping genes, such as for ribosomal protein L7, histone H3.2, histone H2B-2, and amino acid transporter. For the ESTs showing high levels of similarity to previously reported anther-specific genes, RNA blot hybridization was performed to characterize expression patterns of the gene in the anther. Clones 4C3 and 6C1 showed anther-specific expression. 4C3 expressed strongly in the early stage, and 6C1 mainly expressed during the late stage of anther development.

Keywords: anther, cDNA, expressed sequence tag, tobacco

Because of the rapid growth of amino acid sequence information deduced from the nucleotide sequence of cloned genes of known function, possible functions of newly isolated genes frequently can be inferred on the basis of their nucleotide or deduced amino acid sequence homology to genes or gene products of known function (Pearson, 1991). Because reliable, automated DNA sequenators are capable of very high throughput, large-scale partial sequencing of cDNA clones (expressed sequence tags, or ESTs) from humans and several model organisms is now available. EST analysis is an efficient approach for revealing vast amounts of expressed genes in specific organs during certain developmental stages (Lim et al., 1996; Sung et al., 1998).

To date, over 3,700,000 ESTs have been generated from a variety of sources (http:/www.ncbi.nlm.nih.gov /dbEST/dbEST_symmary.html). Over 83% of these belong to the genomes of human (about 50%) and several other animal species, primarily mouse, rat, *Caenorhabditis elegans*, *Drosophila*, and zebrafish. Far fewer ESTs have been reported in plants, with most of the efforts being spent on soybean, maize, tomato, rice, and *Arabidopsis thaliana*.

Tobacco is a very popular model system for studying plant molecular biology and molecular genetics, and has accounted for most of the monumental achievements during the development of plant genetic engineering. Even as transformation technology has advanced, and *A. thaliana* and rice have become the predominant model systems, tobacco is still the single most widely used plant species in the study of molecular developmental biology. To date, however, only 76 ESTs have been reported from *Nicotiana tabacum*.

The anther, at the terminal portion of the stamen, produces pollen and plays a major role in male gametophyte production (Esau, 1977). Anthers have two phases of development. During Phase 1, most of the specialized cells and tissues differentiate, the microspore mother cells undergo meiosis, and tetrads of microspores are formed. During Phase 2, microspores are released from the tetrads, and pollen grain maturation, tissue degeneration, dehiscence, and pollen release take place (Goldberg et al., 1993). The developmental events leading to anther formation and pollen release require coordinated activities among many different cells and tissues, and must occur in a precise, chronological order. However, the molecular mechanisms responsible for the process are not well understood. Each floral organ has its own set of genes; the number of anther-specific genes is estimated to be about 10,000 (Kamalay and Goldberg, 1980, 1984).

To understand the molecular processes in anther development, we analyzed the EST of tobacco anthers to identify any possible homologies with. From 200 ESTs, we found that only 21 ESTs showed meaningful homology.

MATERIALS AND METHODS

Plant Material

Plants of tobacco (*N. tabacum* cv. North Carolina

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82) were grown in a greenhouse at 25 ± 5°C, with a 16-h day length. Flowers were collected at several stages of development, along with leaves and roots. These samples were immediately frozen in liquid nitrogen, and stored at −70°C.

cDNA Library Preparation

Total RNAs were extracted from the anthers during the early developmental stages, from approximately the microspore mother cell stage to the immature pollen stage. Frozen anthers at the expected developmental stage were ground in liquid nitrogen in a mortar with an RNA extraction buffer containing guanidium thiocyanate. The RNAs were then purified via CsCl density gradient centrifugation (Hong and Jeon, 1987). Total RNAs were then passed through an oligo(dT)-cellulose column to extract poly(A)$^+$RNA. cDNA was synthesized using reverse transcriptase and RNaseH (cDNA synthesis kit, Amersham, USA). It was then blunt-end ligated to the SmaI site of pUC18. A cDNA bank was prepared in *Escherichia coli* strain HB101. Clones in the cDNA library were randomly chosen, and cDNA inserts were confirmed by alkaline lysis plasmid miniprep, restriction digest, and agarose gel electrophoresis (Sambrook et al., 1989).

RNA Blot Hybridizations

Total RNAs extracted were run on a 0.8% agarose gel with formaldehyde and blotted onto a nylon membrane. RNA loaded on each lane was normalized to 10 μg, which was confirmed by the measurement of A_{260} and staining of the gel with methylene blue (Sambrook et al., 1989). An RNA ladder (BRL, USA) was used for size markers. Each cDNA clone was labeled with [alpha-^{32}P]dCTP by the random priming method, using a Prime-a-Gene kit (Promega, USA), and then used for RNA blot hybridizations. Hybridizations were carried out in a solution of 5X SSPE, 5X Denhardt's solution, 0.1% SDS, and 100 μg/mL denatured salmon sperm DNA, at 65°C overnight. Afterward, the membrane was washed with 0.2X SSPE and 0.1% SDS at 65°C, and the blot was exposed to an X-ray film with two intensifying screens (DuPont, USA) for two days at −70°C (Sambrook et al., 1989).

Polymerization Chain Reaction

First-strand cDNA was synthesized using MMLV reverse transcriptase for RT-PCR, according to the manufacturer's instruction (Gibco, USA). PCR on the first cDNA was carried out using forward and reverse primers that were complementary to the sequences of the cDNA clones. Thirty-five reaction cycles were used, with each cycle consisting of 30 s at 94°C, 1 min at 48°C, and 40 s at 72°C. For the genomic DNA-PCR, 2 μg of extracted tobacco genomic DNA (Junghans and Metzlaff, 1990) was added with the same primers used for RT-PCR, and the reaction was carried out as RT-PCR. A 1-kb DNA ladder (BRL, USA) was used for size markers (Sambrook et al., 1989).

Nucleotide Sequencing and Homology Analysis

A double-stranded cDNA insert over 400 bp long in pUC18 was sequenced unidirectionally. We used the oligo(dT) from the synthesis of cDNA as a primer, and followed the dideoxy chain termination method (Sanger et al., 1977) with a USB Sequenase 2.0 kit (United States Biochemicals, USA). Sequences were edited manually to remove vector and ambiguous sequences from the ends. The amino acid sequences were deduced from the nucleotide sequence in all six possible frames, and homology was compared using the BLASTX program (Altschul et al., 1990) from NCBI. To achieve the most matches, we conducted multiple alignments of the amino acid sequences, using the CLUSTALW version 1.7 program (Higgins and Sharp, 1988). BLOSUM62 scores >80 were considered significant after regions were selected that showed significant homologies between the amino acid sequences.

RESULTS AND DISCUSSION

Two hundred cDNA clones in a tobacco cDNA library were randomly chosen and partially sequenced. From the 200 sequences, eight showed overlapping sequences to other clones. Thus, nucleotide sequences of 192 clones were searched for homology to genes in the databases, at the amino acid sequence level. Twenty cDNA clones (Table 1) had similarity scores >80, which can be considered significant in homology (Hong et al., 1998). Despite its low similarity score, clone 6C1 was also included because it has shown conserved cysteine residues of lipid transfer proteins (Kader, 1997).

Most of the ESTs showing homology to the reported sequences in the databases belonged to the so-called housekeeping genes. Two cDNA clones showing

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1                                                    TTTAGTTTTCTTGCAATAATCA
23  TTTTGAGTAAGAACTATTCGACCAAATATGGCTACGACCATGAAGTCAGTTGTTTCACTA
                                    M  A  T  T  M  K  S  V  V  S  L  11
83  TGGCTGTTGGGTATGCTGGTGATTGTGCTACAAAGCAGAGTGATTGAATGTCAGCAGGGG
     W  L  L  G  M  L  V  I  V  L  Q  S  R  V  I  E  C  Q  Q  G  31
143 CAGACATGCTCGGCATCACTTGGGAACCTGAACGTGTGCGCGCCTTTCGTGGTGCCAGGG
     Q  T  C  S  A  S  L  G  N  L  N  V  C  A  P  F  V  V  P  G  51
203 GCACCTAACGCCAGCGCTGAGTGTTGTGCTGCACTTCGATCAATTGATAATGACTGCATG
     A  P  N  A  S  A  E  C  C  A  A  L  R  S  I  D  N  D  C  M  71
263 TGTAACACTATGCGCATCTCTGCCCGCCTTCCCTCTCAGTGCAACCTTCCTCCCCTTTCT
     C  N  T  M  R  I  S  A  R  L  P  S  Q  C  N  L  P  P  L  S  91
323 TGTGCTGCAAACTGAGTTTGAAGGGGTCCGCCGTACCAGTCCCAGTCACTGGCGAATAAT
     C  A  A  N  *                                                95
383 ATCATAGTTCTAAAATCAATAAATTTACTCTGTCCCTTCTATCTTTCTTTCCCTTTTTTC
443 CGTGGTGCTCTTTATGCTAATGTCCTCACTCCTCTGTTCTGTCACAGAGCAAGGCCAGGA
503 AAGAGTTTGTATTGTCATATGAAATCAATAAAACATGTTTGTTCGTCAAAAAAAAAAAAA
563 AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. Nucleotide sequence of 4C3, and the deduced amino acid sequence. Putative open reading frame is shown below the nucleotide sequence. Putative translation termination codon is marked by an asterisk. Putative poly(A) additional signal sequences are in italics. Sequences underlined are the primer binding sites for RT-PCR and genomic DNA-PCR.

homology to genes previously reported as anther-specific were chosen for further analyses. Clone 4C3 showed high homology to Protein 108 precursor, and clone 6C1 had conserved cysteine residues of lipid transfer proteins. Both genes showed complex regulation patterns in gene expression, but they were also shown to be anther-specific during the development of flowering plants (Smith et al., 1990; Kader, 1997). These clones were fully sequenced, and a similarity search was performed again using BLASTX.

4C3 coded a protein of 95 amino acids (Fig. 1), and was a homolog of protein 108 precursor which shows tapetum-specific expression (Smith et al., 1990). It also shared similarity with several proteins specifically expressed in the anther (Fig. 2), such as the A9 protein in *A. thaliana* and *Brassica napus* and the FIL protein in *Antirrhinum majus* (Nacken et al., 1991; Roberts et al., 1991; Paul et al., 1992).

6C1 coded a protein of 120 amino acids (Fig. 3), and showed similarity to the LTPs (lipid transfer proteins), although the similarity was not high. Diverse functions of LTPs have been reported, including cutin biosynthesis, wax formation, pathogen-defense reactions, and environmental stress responses. Structural variation of LTPs is thus considered necessary to accommodate these diverse functions (Kader, 1997). But all the LTPs reported so far have conserved cysteine residues, and 6C1 showed eight perfectly conserved cysteine residues in place (Fig. 4). Interestingly, 4C3 was also observed with the perfectly conserved

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4C3             MATTMKSVVSLWLLGMLVIVLQSRVIECQQGQTCSASLGNLNVCAPFVVP---GAP--NA
tomato          SSSSSSSFISLLLLILLVIVLQSQVIECQPQQSCTASLTGLNVCAPFLVP---GSP--TA
Snapdragon-1    ----MKSIVP--LVMLTVLVAQSQLITQSEAQTCSASLANLNACAPFVVL---GAAT-TP
arabidopsis     -MVSLKSLAA-ILVAMFLATG-----PTVLAQQCRDELSNVQVCAPLLLP---GAVNPAA
rapeseed        ----LKSFTT-ILFVMFLAMSALETVPMVRAQQCLDNLSNMQVCAPLVLP---GAVNPAP
maize           ------------------------------QTCAGQLRGLAPCLRYSVPPLPGQVPPAP
                                               * *  .* .:  *    :    *     .

4C3             SAECCAALRSIDNDCMCNTMRISARLPSQCNLPPLSCAAN
tomato          STECCNAVQSINHDCMCNTMRIAAQIPAQCNLPPLSCSAN
Snapdragon-1    SSDCCTALQSVDHECLCNTLRIASRVPAQCNLPPLSCGAN
arabidopsis     NSNCCAALQATNKDCLCNRLRAATTLTSLCNLPSFDC---
Rapeseed        NSNCCIALQATNKDCICNALRAATTFTTTCNLPSLDC---
maize           GPECCSALGAVSRDCACGTFSIINSLPAKCALPPVSC---
                ..:** *: : ..:* *. :      ..: * **...*
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Figure 2. Homology comparison of 4C3 with other proteins in the databases: tomato, tomato protein 108 precursor; snapdragon-1, snapdragon fil1 protein; arabidopsis, arabidopsis tapetum-specific protein A9 precursor; rapeseed, rapeseed tapetum-specific protein A9 precursor; maize, maize anther-specific protein MZM3-3 precursor. An asterisk indicates the position with identical amino acid. ":" represents the position with highly similar amino acid. "." represents the position with similar amino acid.

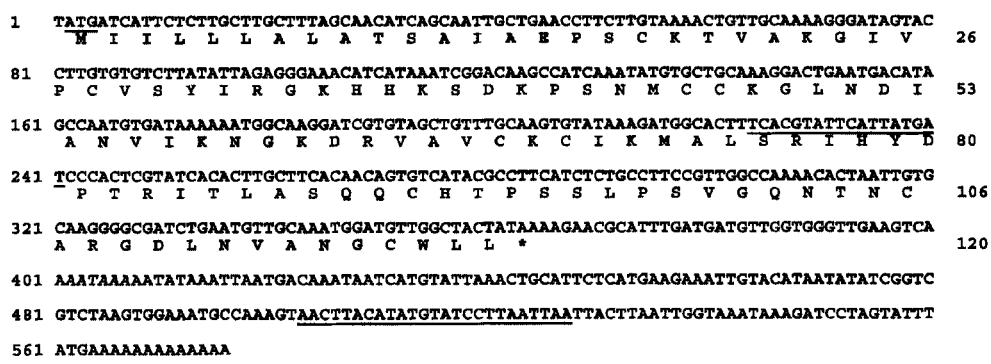

Figure 3. Nucleotide sequence of 6C1, and the deduced amino acid sequence. Putative open reading frame is shown below the nucleotide sequence. Putative translation termination codon is marked by an asterisk. Putative poly(A) additional signal sequence is in italics. Sequences underlined are the primer binding sites for RT-PCR and genomic DNA-PCR.

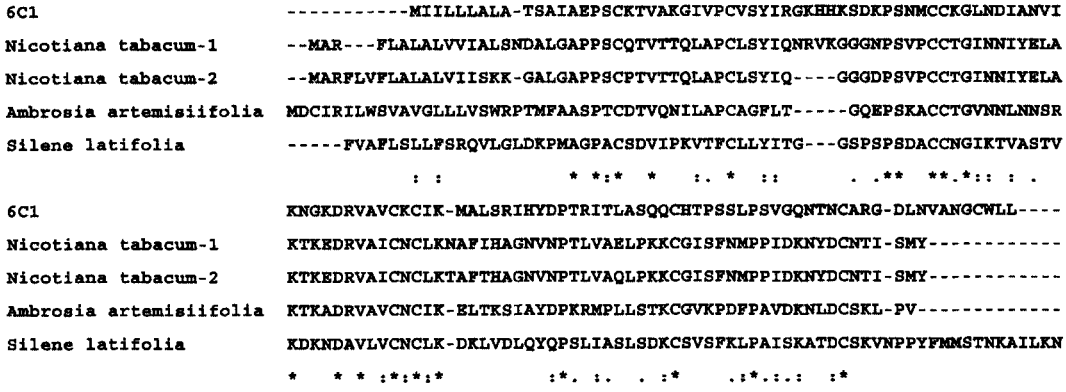

Figure 4. Homology comparison of 6C1 with other proteins in the databases: Nicotiana tabacum-1, *N. tabacum* lipid transfer protein (clone ant43C); Nicotiana tabacum-2, *N. tabacum* lipid transfer protein (clone ant43D); Ambrosia artemisiifolia, *Ambrosia artemisiifolia* allergen Amb a VI; Silene latifolia, *Silene latifolia* Men-7. An asterisk indicates the cysteine residue conserved among the proteins. ":" represents the position with highly similar amino acid. "." represents the position with similar amino acid.

eight cysteine residues with other homologous proteins specifically expressed in the anther (Foster et al., 1992) (Fig. 2), although the speculated functions of 4C3 and 6C1 could be quite different. Because spatial expression pattern of both genes could be similar, possibly related functions of both proteins might be suggested.

RNA blot analysis for 4C3 expression confirmed that it is anther-specific (Fig. 5A). RT-PCR for the transcript of 4C3 also demonstrated its anther-specific expression (Fig. 5B). The temporal expression pattern of 4C3 showed that the gene is turned on early in anther development. The transcript level was maintained until the tetrad stage and, thereafter, rapidly declined (Fig. 5C). PCR of the genomic DNA for a portion of 4C3 showed a band of 300 bp, the exact size of DNA expected to be produced from the designed primers (Fig. 5E). Thus, genomic DNA-PCR confirmed the presence of 4C3 in the genome of tobacco with the nucleotide sequence shown in Figure 1.

RNA blot analysis for the expression of 6C1 confirmed that 6C1 is also anther-specific (Fig. 6, A and B). RT-PCR for the transcript of 6C1 again confirmed its anther-specific expression (Fig. 6B). The temporal expression pattern of 6C1 showed that the gene is turned on in the middle of anther development. The transcript started to show up at the tetrad stage, and had strongly accumulated by the microspore stage. Thereafter, 6C1 transcript could not be detected (Fig. 6C). PCR of the genomic DNA for a portion of 6C1 showed a band at 560 bp, which was larger than was expected to be produced from the designed primers (Fig. 6E). This size discrepancy might indicate the presence of intron between the sites used for binding the primers for PCR. We believe that the results of RNA blot analyses, RT-PCR, and genomic DNA-PCR

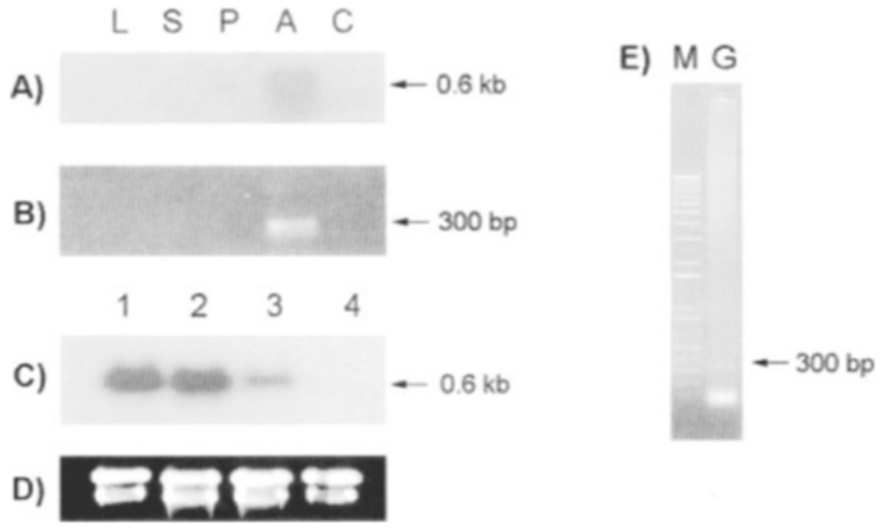

Figure 5. Expression pattern of 4C3 in the anther of *N. tabacum* cv. NC82. A) Total RNA was extracted from leaf, sepal, petal, anther, and carpel, and subjected to RNA blot hybridization with the ^{32}P-labeled 4C3 clone. B) All the RNA samples used for RNA blot in A) were subjected to RT-PCR based on the nucleotide sequence of 4C3. C) Total RNA was extracted from the anther at several developmental stages, and subjected to RNA blot hybridization with the ^{32}P-labeled 4C3 clone. D) Ethidium bromide staining of a duplicate RNA gel of C). E) PCR of tobacco genomic DNA for a portion of 4C3. L, leaf; S, sepal; P, petal; A, anther; C, carpel; 1, anther at the stage of microspore mother cell; 2, anther at the stage of tetrad; 3, anther at the stage of released microspore; 4, anther at the stage of immature pollen; M, 1-kb DNA ladder (BRL, USA); G, PCR product.

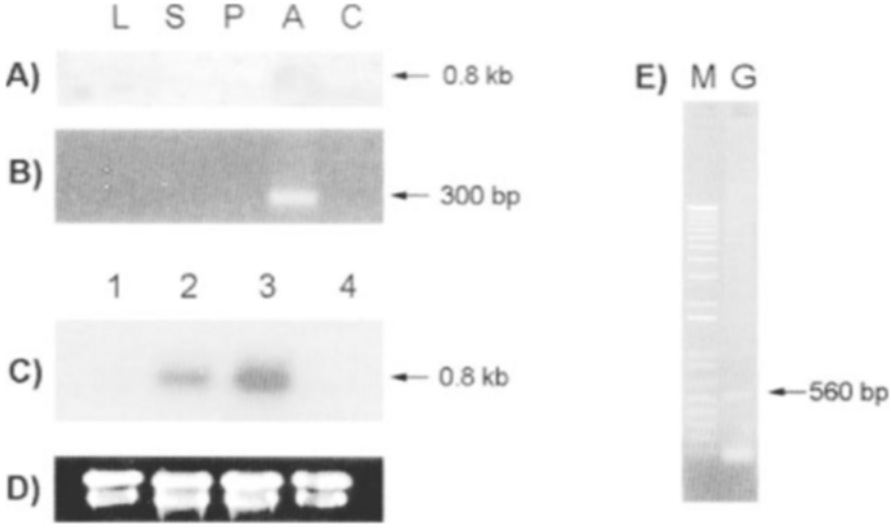

Figure 6. Expression pattern of 6C1 in the anther of *N. tabacum* cv. NC82. A) Total RNA was extracted from leaf, sepal, petal, anther, and carpel, and subjected to RNA blot hybridization with the ^{32}P-labeled 6C1 clone. B) All the RNA samples used for RNA blot in A) were subjected to RT-PCR based on the nucleotide sequence of 6C1. C) Total RNA was extracted from the anther at several developmental stages, and subjected to RNA blot hybridization with the ^{32}P-labeled 6C1 clone. D) Ethidium bromide staining of a duplicate RNA gel of C). E) PCR of tobacco genomic DNA for a portion of 6C1. L, leaf; S, sepal; P, petal; A, anther; C, carpel; 1, anther at the stage of microspore mother cell; 2, anther at the stage of tetrad; 3, anther at the stage of released microspore; 4, anther at the stage of immature pollen; M, 1-kb DNA ladder (BRL, USA); G, PCR product.

(Figs. 5 and 6) sufficiently demonstrate that the ESTs produced in the anther library properly represent the transcripts in the anther.

EST analysis is an efficient approach for revealing vast amounts of expressed genes in specific organs during certain developmental stages (Lim et al., 1996; Sung et al., 1998). The lack of EST analysis, to date, for the anthers of flowering plants is probably the reason for the low number of ESTs showing homology to previously reported genes. Therefore the study of anther

Table 1. Similarities of the cDNA clones from tobacco anther to the known genes in the databases. Clone represents laboratory identifications. Length compared represents the number of amino acid residues between a query sequence and its matched protein. % match means percent identity at the amino acid level. Score represents BLOSUM62 value presented by NCBI. Dbj, DNA database of Japan; sp, SwissProt; prf, Protein Research Database; gb, GenBank; pr, Protein Information Resource; emb, European Molecular Biology Lab.

Clone	Putative identification	Source of comparison	Length compared	% match	Score
1A11	Light harvesting chlorophyll a/b binding protein	*Nicotiana sylvestris* (dbj)	60	86	108
1C1	MCM2-related protein	*Arabidopsis thaliana* (emb)	79	54	88
2A3	ADH1_alcohol dehydrogenase 1	*Petunia x hybrida* (sp)	81	96	169
2A8	Ribosomal protein L7	*Solanum tuberosum* (prf)	57	98	124
2D4	T3P18.8	*Arabidopsis thaliana* (gb)	58	75	82
2D5	H32_MEDSA histone H3.2	*Arabidopsis thaliana* (sp)	68	98	136
3B6	Heat shock protein MTSHP precursor	*Lycopersicon esculentum* (pir)	145	57	137
3C6	Histone H2B-2	*Lycopersicon esculentum* (pir)	55	89	90
3D8	Aspartic proteinase homolog F17M5.250	*Arabidopsis thaliana* (pir)	99	44	90
3D10	Cytochrome c oxidase subunit 5c-like protein	*Arabidopsis thaliana* (emb)	63	77	111
4C3	108_LYCES Protein 108 precursor	*Lycopersicon esculentum* (sp)	100	63	141
4C10	NTF4_TOBAC mitogen-activated protein kinase homolog NTF4	*Nicotiana tabacum* (sp)	61	96	125
4E6	Putative DNA-directed RNA polymerase subunit	*Arabidopsis thaliana* (gb)	105	44	98
5G7	Catechol oxidase precursor	*Nicotiana tabacum* (pir)	90	67	116
6C1	Lipid transfer protein (clone ant43D)	*Nicotiana tabacum*-1 (pir)	64	34	53
6D3	Putative protein phosphatase 2C	*Arabidopsis thaliana* (gb)	55	74	99
6E5	40S Ribosomal protein S8	*Prunus armeniaca* (gb)	85	56	84
6G12	Ribosomal protein L18a	*Arabidopsis thaliana* (pir)	66	86	125
7A3	Probable amino acid transfer protein AAP2	*Solanum tuberosum* (pir)	41	87	80
7A5	Cytosolic heat shock 70kD protein	*Solanum oleracea* (gb)	81	81	127
10A5	Putative proline-rich protein	*Arabidopsis thaliana* (gb)	63	66	92

development at the molecular level is just at the beginning stage.

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