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דו"ח דיווח מדעי
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נושא המחקר: בידוד ואפיון פרומוטרים צמחיים ליצירת צמחים טרנסגנים בעלי חשיבות חקלאית

סוג דו"ח : דו'ח מדעי סופי

חוקר ראשי : זלץ יחיעם

חוקרים משניים: ויינשטיין אלכסנדר
לוינסון אפרים
ברג רבקה
קפולניק יורם
פירון נורית
גרנות דוד

מקורות מימון עבורם מיועד הדו"ח:

משרד המדע

תקציר הדו"ח:

מטרת מחקר זה הינה בידוד ואפיון פרומוטורים צמחיים אותם ניתן יהיה לנצל ביצירת צמחים טרנסגניים בעלי חשיבות חקלאית.
תנאי הכרחי ליצירת הצמחים הטרנסגניים הינו זיהוי בידוד ואפיון פרומוטורים צמחיים המתבטאים באופן ייחודי ברקמות הצמח השונות כדי שניתן יהיה לכוון את ביטוי הטראנסגן לרקמה המתאימה. הפרומוטורים אותם בחרנו לאפיין הינם כאלה הנדרשים לטיפוח צמחי מאכל ונוי, עקב התבטאותם באברי צמח בעלי חשיבות כלכלית מרובה:1 . פרי הצעיר;2 . עלי-כותרת;3 . מאבקים. על מנת לאפשר בידוד שבטי cDNA יצרנו שתי ספריות cDNA: האחת מפרחים שנאספו בגילים שונים והשניה מפרי פרתנוקרפי צעיר (10 ימים לאחר אנתזיס). בידוד cDNAs יחודיים לאיברי הצמח הנ"ל נעשה ע"י יצירת ספריות מופחתות ומושוות של cDNA representations. איפיון השבטים ע"י ריצופם ובחינת התבטאותם מראה שבידינו 10 רצפים ייחודיים לפרי צעיר ולשחלה,18 רצפים ייחודיים למאבק,6 רצפים ייחודיים לעלי-כותרת ו-13 רצפים המתבטאים במאבקים ובעלי הכותרת ואינם מתבטאים באברים אחרים של הצמח.
בידדנו וריצפנו שבטי cDNA המייצגים פרומוטרים יחודיים לכל אחד מאברים הנ"ל, זיהינו שבטי BAC המכילים את הגן המייצר את ה-cDNA וקבענו את הרצף של הדנ"א הגנומי הכולל את הפרומוטרים של שלושה גנים שרצפיהם אינם מופיעים ב-GeneBank ובפרוייקט ה-EST של העגבנייה:
I. yfe17 המייצג myb-like gene ייחודי המתבטא רק בפרי צעיר
II. fle186 המייצג ORF שהפאראלוג שלו ב-Lilium מתבטא אך ורק בתאי האם של גרגרי האבקה
III. fle38 המייצג GDP dissociation inhibitor המתבטא באופן ייחודי בעלי הכותרת
את רצף הפרומוטר הכולל 1030 בסיסים מהגן fle38 1200 בסיסים מהגן fle186 ו-500 בסיסים של הגן yfe17 חיברנו לגן המדווח gusA בפלסמיד בינארי של Agrobacterium ועתה אנו מייצרים צמחי טבק, ארבידופסיס ועגבנייה מותמרים במבני הדנ"א הנ"ל.

חתימות ואישורים:

חוקר ראשי	מנהל המחלקה	מנהל המכון	אמרכלות	תאריך
				23.7.01

Final Report to MOST – Project No. 9446

Characterization of tissue specific promoters for the manipulation of useful agricultural traits in transgenic plants

Y. Salts, R. Barg, A. Vainstein, Y. Kapulnik, E. Lewinsohn, D. Granot,

N. Firon, M. Flaishman

Objective and importance of the proposal

The objective of this proposal is to isolate and characterize promoters that are highly specific either to the ovary and young fruit, or to anthers or to petals.

Since these organs are of significant agronomic importance, hence isolation of promoters that are uniquely expressed in each of these organs is of a high practical value. Ovary and young fruit specific promoter can be utilized to affect fruit nutritional value, fruit set efficiency, its shape and size, sugar and starch content and composition (e.g. Trethewey & Willmitzer 1999), organic acids content and composition, and the content of different aromatic compounds which affect the fruit's aroma and flavor. Petal specific promoters can be exploited in order to affect flower color (e.g. Elomaa et al. 1996), petal shape, scent, and flower shelf-life longevity, all are attributes which are highly valuable for the industry of ornamental flowers. Anther specific promoter can be utilized to induce male sterility (e.g. Mariani et al. 1990, 1992) which is an important trait for several purposes: In many crop plants it enables efficient hybrid seeds production; in flowering plants it can prolong the vase-life since pollination induces flower senescence, and in many ornamentals and grasses it will prevent the undesirable pollen-allergenic effects.

The ability to perform such manipulations depends on the availability of promoters that are highly specific to each of the above mentioned plant organs. Such promoters are scarce, and most of them are protected by patents, hence not accessible to Israeli scientists who wish to perform applied research in either the industry or academic institutes.

Results of the work performed in the previous years

In the present study tomato is being used as the model plant.

In order to enable isolation of full-length cDNA clones of the organ specific genes, we constructed two cDNA libraries: one from flowers harvested at different developmental stages (from pre-anthesis to mature open flowers), and one from young parthenocarpic (seedless) fruits. That, to prevent representation of seed specific clones in the fruit derived library. Both libraries are of high quality, containing about 10^6 clones, most of which represent full-length cDNAs. The libraries were deposited in the Plant Genome Center at The Weizmann Institute of Science, and full length cDNAs of genes of interest to different Israeli scientists were cloned from these libraries (e.g. auxin binding protein 1, fructokinase, malate-dehygrogenase, *FIE* and *TKn1* by R Barg & Y Salts, D Granot, A Sadka, N Ohad, and G Grafi respectively).

Identification of tissue-specific promoters was performed by the isolation of organ specific cDNA representations, utilizing CLONTECH's "PCR-Select™ cDNA Subtraction Kit" (Gurskaya et al 1996). This methodology was employed to generate two different representations libraries: One library is derived from young parthenocarpic tomato fruit, and the second library is composed of representations derived from petals and stamens.

PCR products harboring representative inserts of 160 of the young fruit library were arrayed on nylon membranes and hybridized with radio-labeled cDNAs derived from the tester mRNA (young parthenocarpic fruit) and the driver mRNA (seedlings + petals + stamens). About 70% of the clones were found to be preferentially expressed in fruit. We sequenced 44 of the fruit expressed clones and found that they represent 21 unique sequences presented in Table 1. Northern analyses of 10 of these clones which exhibit ovary and young fruit specificity are presented in Fig. 1. These genes vary in their temporal pattern of expression and promoter strength.

Following a similar procedure, a cDNA representations library was constructed from tomato petals and stamens which served as the source of the tester mRNA, while a mixture of mRNAs from whole seedlings with 4 true leaves, ovaries at pre- and post-anthesis and young seeded fruit (2.5 cm in diameter), served as the driver. From this library 800 clones were arrayed on nylon membranes and hybridized with radio-labeled cDNA derived from the tester and the driver. Only 20% of the clones were found to hybridize to the driver cDNA, indicating that the

subtraction procedure was highly effective. These clones represent 91 unique cDNA sequences, as determined by sequencing and hybridization (see Table 2). The 23 sequences - which are marked by an asterisk in Table 2 - exhibit homology to known pollen and/or stamen specific genes in GeneBank, again indicating the high quality of our subtracted library.

Fifty-five of our unique flower-expressed-sequences are not present in either GeneBank or the tomato 90,346 EST sequences released by TIGR on August 4, 2000, which represent 23,220 unique sequences (http://www.tigr.org). This indicates that our methodology for identification of organ specific cDNAs is highly cost effective and economically superior to the very expensive EST project.

Northern analysis was performed on 43 of the clones which hybridized only to the driver DNA and for which expression pattern could not be predicted by homology to pollen or anther expressed genes. This analysis revealed that 41 out of the 43 clones exhibit organ specific expression and only 2 clones showed non-specific expression. We found that the expression of 20 of the genes is restricted to the stamens, 6 clones are almost exclusively expressed in petals, 14 are expressed in petals and stamens but not in other plant organs, and one is restricted to petals and young fruit (Table 2).

Northern analysis of a sample of seven clones highly specific to stamens and six petal specific clones are shown in Fig. 2 and Fig. 3 respectively.

The two collections (fruit & flower) of the unique representative clones were supplied to Prof. R Fluhr and Prof. J Hirschberg to be included in the Israeli tomato microarray.

We concentrate our efforts on the promoters of three organ specific genes which harbour either young fruit, stamen or petal specific promoters (*yfe17*, *fle186* and *fle38* respectively).

Since each of the genes could be a member of a large gene family (as two of them definitely are), we sequenced cDNA clones representing these genes, and utilized specific probes derived from the 3' or 5' untranslated regions (UTR) to identify tomato BAC (bacterial artificial chromosome) clones harbouring each of the three organ specific genes.

The work performed with each of the genes is detailed below:

1. Young fruit expressed gene *yfe17* (see Fig. 1)

We determined the full length sequence of *yfe17* cDNA and deposited it at EMBL (accession AJ277944). It encodes a *myb*-like protein harbouring only one DNA binding domain, and contains 77 nucleotides at its 5' untranslated region. It is highly expressed in young fruit at the stage in which fruit development is driven mainly by cell division, unlike later stages where cell enlargement is the main mechanism for fruit expansion.

A BAC which contains *yfe17* has been identified, and we determined the genomic sequence of 3604 bp which include 2430 bp upstream of the transcription initiation point. The genomic sequence indicates that *yfe17* contains an intron within the 3' untranslated region. 1200 bp of this sequence (which contains the 5'-UTR) were fused to the *gusA* gene in an Agrobacterium binary vector and this construct is being transformed into tomato, tobacco and Arabidopsis.

Stamens expressed gene *fle186* (Fig. 2)

This gene encodes a protein that is homologous to a *Lilium* cDNA which is expressed only in the microspore mother cells at the zygotene stage of meiotic prophase (Kobayashi et al. 1994). We isolated and sequence the full length cDNA of this gene. This sequence served identify a BAC that contains *fle186*. From this BAC we isolated the gene and determined the genomic sequence of 3117 bp which include 1726 bp upstream of the transcription initiation point. The genomic sequence indicates that *yfe17* contains four introns. 500 bp of this sequence (which contains the 5'-UTR) were fused to the *gusA* gene in an Agrobacterium binary vector and this construct is being transformed into tomato, tobacco and Arabidopsis.

Petal expressed gene *fle38* (Fig. 3)

This gene encodes for a GDP dissociation inhibitor (GDI), which is highly expressed in petals, yet scarcely expressed in vegetative or other reproductive organs. We determined the cDNA sequence and deposited it at EMBL (accession AJ401079).

We identified a tomato BAC which contains *fle38*, and sequenced its cDNA and ~7kbp of its genomic DNA which reveals the existence of at least 13 exons and 12 introns (see Fig.4). 1030 bp of this sequence (which contains the 5'-UTR) were fused to the *gusA* gene

in an Agrobacterium binary vector and this construct is being transformed into tomato, tobacco and Arabidopsis.

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Table 1. Independent fruit expressed cDNA representations derived from young parthenocarpic tomato fruit

	Clone #	Length (bp)	Identical ESTs at TIGR 30.8.00	Homology by BLASTN or BLASTX (Score / E value)	Expression Pattern in Northern (Fig 1)*
1	7	489	AI489089	AT glucosyltransferase (88 / 5e-17)	Ovary + young fruit
2	17	570	none	AT unknown protein (107 / 8e-23)	Ovary + young fruit
3	18	260	TC2251	LE 2-oxoglutarate-dependent dioxygenase (354 / 2e-97)	NT
4	20	370	AI487895	AT putative peptide transporter (108 / 2e-23)	Ovary + young fruit
5	21	538	TC39545	N. plumb putative cytochrome P-450 (254 / 3e-67)	Ovary + young fruit
6	23	531	AI895017	AT putative Ckc2, containing AP2 domain (66 / 3e-10)	Stem + ovary + young fruit
7	24	260	TC36677	none	Ovary + young fruit
8	26	640	TC41707	Alfalfa malate dehydrogenase precursor (449 / e-125)	Ovary + young fruit
9	27	552	TC4353	AT hypothetical protein (243 / 9e-64)	Stem + ovary + young fruit
10	32	260	TC1330	LE TPRP-F1	NT
11	37	180	TC41874	Tomato alcohol dehydrogenase homologe GAD3 (345 / 3e-93)	NT
12	46	115	none	none	Not detectable
13	67	290	TC1382	LE superoxide dismutase 2 Cu-Zn (191 / 1e-48)	NT
14	69	429	AI483562	N.plumb 40S ribosomal protein S17 (168 / 2e-41)	Ovary + young fruit
15	73	182	TC36680	Phaseolus zeatin O-glucosyltransferase (69 / 6e-12)	Ovary + young fruit
16	75	640	none	AT putative polygalacturonidase (256 / 9e-68)	NT
17	80	356	TC3848	N. plumb putative cytochrome P-450 (176 / 7e-44)	NT
18	81	>700	AI488035	AT ? Chlamydia predicted pseudouridine synthase (114 / 1e-24)	Ovary + young fruit
19	102	300	TC1368	none	Stem + ovary + young fruit
20	107	240	TC41630	none	Not clear
21	146	452	TC3461	none	Petal+stamen+ovary+young fruit

* NT - not tested

Table 2. Independent flower expressed cDNA representations derived from tomato petals and stamens

	Clone #	Length bp	Identical ESTs at TIGR 30.8.00	Homology by BLASTN or BLASX	Expression Pattern in Northerns
1	2 fl	368	none	none	Petals + stamens
2	4 fl	270	TC8207	Tomato leaf wound-induced proteins inhibitor II	
3	5 fl	546	none	AT putative beta-1,3-endoglucanase (partial)	Stamens
4	**6 fl***	380	none	Olive pollen allergen	Petals + stamens
5	7 fl	120	TC3935	ACYL-COA-BINDING [FRITILLARIA AGRESTIS]	
6	10 fl	468	AI776907	Solanum chloroplast fibrillin	
7	13 fl	210	none	none	Petals + stamens
8	**14 fl***	425	TC24060	Tomato pollen specific LAT59 gene pectate lyase	
9	17 fl	500	AW648909	cDNA expressed in chickpea epicotyls partial	Petals
10	20 fl	443	none	none	Stamens
11	23 fl	181	TC20953	hexose transporter [Vitis vinifera] (see 594 fl)	Stamens
12	25 fl	591	TC949	S. tuberosum beta-fructofuranosidase	
13	27 fl	541	none	none	Petals + stamens
14	28 fl	512	none	none	Stamens
15	29 fl	428	none	AT DNA-DAMAGE-REPAIR protein	Petals
16	**33 fl***	278	none	LIM7 induced in meiotic prophase in lily microsporocytes 3' end (extension of 186)	Stamens
17	**35 fl***	550	none	germinating petunia pollen - leucie rich repeat protein	Stamens
18	**36 fl***	429	none	monosaccharide transporter 1 [Petunia] pollen specific	
19	38 fl	321	none	N. tab GDP dissociation inhibitor	Petals
20	43 fl	440	none	zinc finger protein [Homo sapiens]	Stamens
21	44 fl	200	AI772624	Tomato abscisic stress ripening protein 1	
22	46 fl	283	none	none	Stamens
23	51 fl	430	TC8600	L.esculentum LEACO2 (ACC oxydase)	
24	54 fl	490	TC632	NONSPECIFIC NT LIPID-TRANSFER PROTEIN 2 LTP2)	
25	61 fl	320	TC3503	dormancy related protein [Trollius ledebourii]	
26	65 fl	351	none	none	Petals + stamens
27	67 fl	597	none	AT pectinacetylesterase	Petals + stamens
28	**69 fl***	396	none	Rape pollen expressed arabinogalactan protein	Petals + stamens
29	70 fl	610	none	F1N21.11 hypothetical [Arabidopsis thaliana]	
30	71 fl*	235	none	Tobacco pollen-specific polygalacturonase	
31	73 fl	278	TC5227	translation initiation factor 6 [Homo sapiens]	
32	74 fl	527	AI486368	LeMir soybean Kunitz trypsin inhibitor family (partial)	
33	**80 fl***	410	TC21639	NTP303 pollen specific mRNA	
34	97 fl	>800	none	LEA PROTEIN - CICER ARIETINUM (CHICKPEA)	
35	**98 fl****	440	**none**	pistil expressed gamma-thionin homolog [Petunia inflata]	
36	111 fl	418	none	none	Stamens
37	118 fl	432	none	none	Stamens
38	**122 fl***	465	none	maize pectin methylesterase-like gene, ZmC5, pollen expressed	Stamens
39	126 fl	255	none	none	Petals (weak)
40	**128 fl***	699	none	pectate lyase (EC 4.2.2.2) LAT59 - tomato (not identical)	
41	130 fl	248	TC2520	alpha-expansin precursor [Nicotiana tabacum]	
42	134 fl	157	AW623609	none	non-specific
43	**146 fl***	533	none	pollen expressed pectinesterase [Petunia inflata]	
44	148 fl	279	none	none	Stamens
45	151 fl	360	none	Solanum tuberosum actin (Pot82) gene	
46	155 fl	664	none	fructokinase [Lycopersicon esculentum]	Stamens
47	169 fl	340	none	cupredoxin, CPC=type I copper protein [Cucumis sativus]	

Homologous to known pollen or anther expressed genes (**bold** letters)

* pistil expressed gene (**bold** letter)

Table 2 (continued). Independent flower[redacted]ressed cDNA representations derived from tomato [redacted]ls and stamens

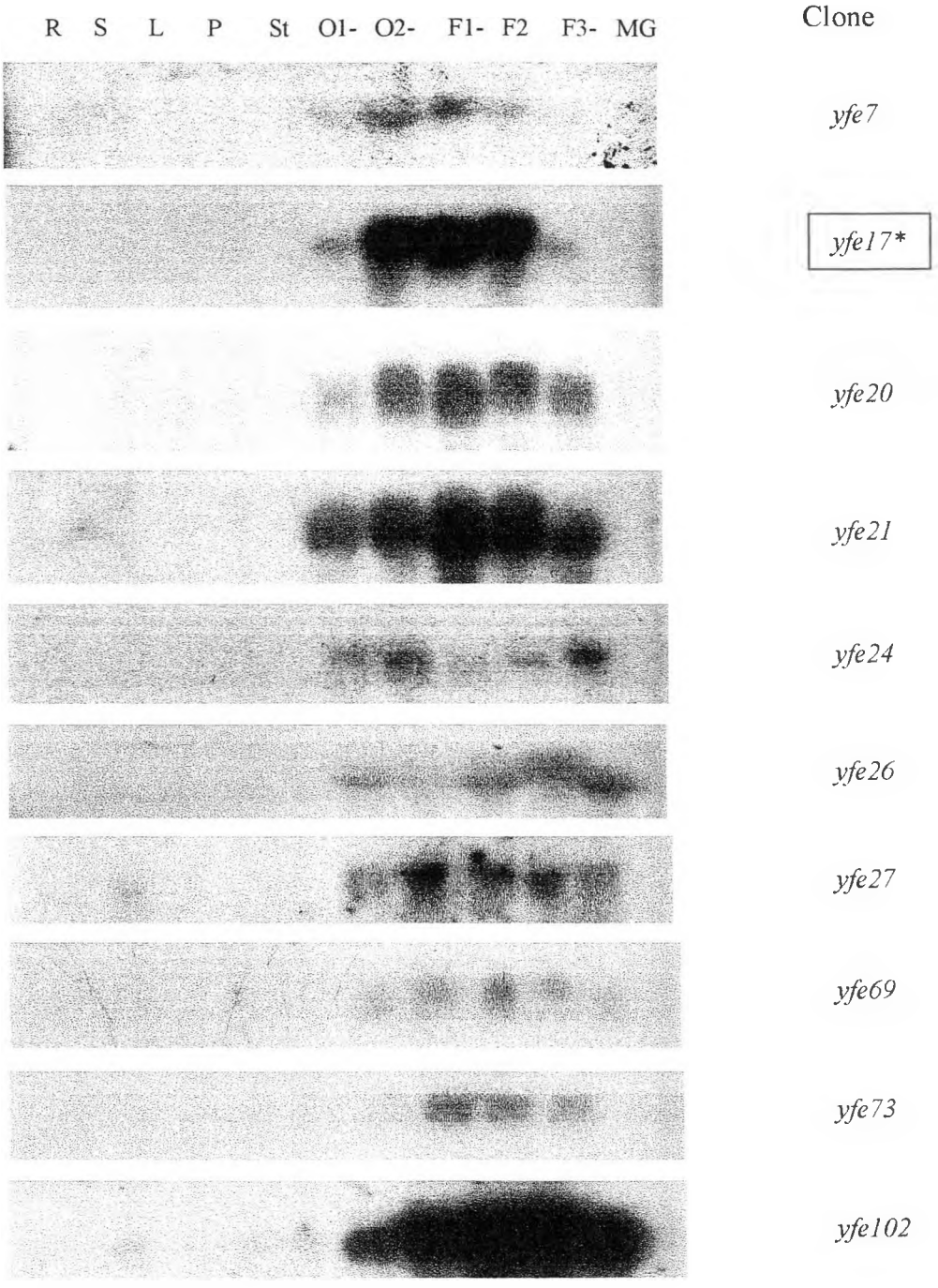
	Clone #	Length bp	Identical ESTs at TIGR 30.8.00	Homology by BLASTN or BLASX	Expression Pattern in Northerns
48	177 fl	97	TC8207	Tomato leaf wound-induced proteinase inhibitor II	
49	**186 fl***	580	TC19725	LIM7 induced in meiotic prophase in lily microsporocytes 5' end (extension of 33)	Stamens
50	192 fl	740	none	copper protein umecyanin [horseradish, roots]	
51	**200 fl***	650	none	PGP177 petunia germinating pollen expressed	
52	202 fl	311	none	putative beta-galactosidase [Arabidopsis thaliana]	Stamens
53	**214 fl***	214	none	Tomato mature pollen-specific receptor-like protein kinas	
54	268 fl	350	none	cortexillin I [Dictyostelium discoideum]	
55	**280 fl***	227	TC47248	Petunia pollen specific protein PGP301	Stamens
56	**316 fl***	414	none	Arabidopsis anther-specific H+-transporting ATPase	
57	323 fl	680	none	Similar to Arabidopsis protein kinase APK1A	Stamens
58	347 fl	680	AI772162	cytochrome P450 [Nicotiana tabacum]	
59	**364 fl***	383	none	Tobacco pollen-specific polygalacturonase 3' end	
60	**397 fl***	346	none	germinating petunia pollen PGP301	
61	401 fl	192	TC6716	Tomato wound-induced proteinase inhibitor I	
62	410 fl	315	AI773899	phenylalanine ammonia-lyase 2 [Arabidopsis thaliana]	
63	428 fl	568	AW040930	Unknown protein [Arabidopsis thaliana]	Petals
64	**431 fl***	320	TC45019	Tomato LAT56 gene for protein P56 pectate lyases homologue	
65	443 fl	192	none	none	
66	449 fl	381	TC3369	auxin-induced protein [Synechocystis sp.] (Aldose reductase)	Petals
67	**454 fl***	696	none	Tomato nther specific LAT52 (not identical)	
68	**455 fl***	540	BE354381	Tomato anther specific LAT52 gene 3' end	
69	475 fl	277	none	none	Petals + stamens
70	**477 fl***	511	none	PGP177 germinating petunia pollen	
71	479 fl	243	TC19905	similar to arabinogalactan protein precursor	
72	525 fl	280	none	none	Stamens
73	535 fl	270	AI487167	none	
74	538 fl	450	none	none	Stamens
75	551 fl	517	TC9832	Lycopersicon esculentum APETALA3 homolog LeAP3	
76	**564 fl***	320	none	pollen expressed pectinesterase [Petunia inflata]	
77	565 fl	571	none	chloroplast carbonic anhydrase [Nicotiana tabacum]	
78	**568 fl***	179	TC37419	Tobacco POLLEN-SPECIFIC PROTEIN NTP303 (ASCORBASE)	
79	582 fl	530	none	putative calmodulin [Arabidopsis thaliana]	
80	596 fl	343	none	Tomato LAT59 gene for protein P59 (putative pectate lyase)	
81	673 fl	640	AI484755	cathepsin B-like cysteine proteinase [Nicotiana rustica]	
82	683 fl	362	AW624042	geranylgeranyl pyrophosphate synthase [Scoparia dulcis]	
83	685 fl	460	TC44300	GTP-binding protein [Arabidopsis] (RAB6 isoform, human)	non-specific
84	686 fl	240	none	hypothetical protein [Arabidopsis thaliana]	Petals + stamens
85	699 fl	545	none	putative LIM-domain protein [Arabidopsis thaliana]	Petals + stamens
86	701fl	317	none	serine proteinase inhibitor - potato	Petals + young fruit
87	708 fl	247	TC20462	Extension of 795 fl hypoth. prot [Arabidopsis & Synechocystis]	
88	718 fl	425	none	none	Petals + stamens
89	**730 fl***	620	none	Pollen specific membrane integral protein - [N. alata]	
90	752 fl	237	none	CDK5 kinase [Drosophila melanogaster]	Stamens
91	755 fl	>650	TC20867	hypothetical protein [Arabidopsis thaliana]	Petals + stamens
92	794 fl	361	TC23285	pectinesterase [Petunia inflata]	Petals + stamens
93	795 fl	406	TC20462	Extension of 708 fl. hypoth. prot [Arabidopsis & Synechocystis]	Petals + stamens

Homologous to known pollen or anther expressed genes (**bold** letters)

* pistil expressed gene **(bold** letter)

Figure 1. Expression patterns of different young fruit expressed cDNA clones

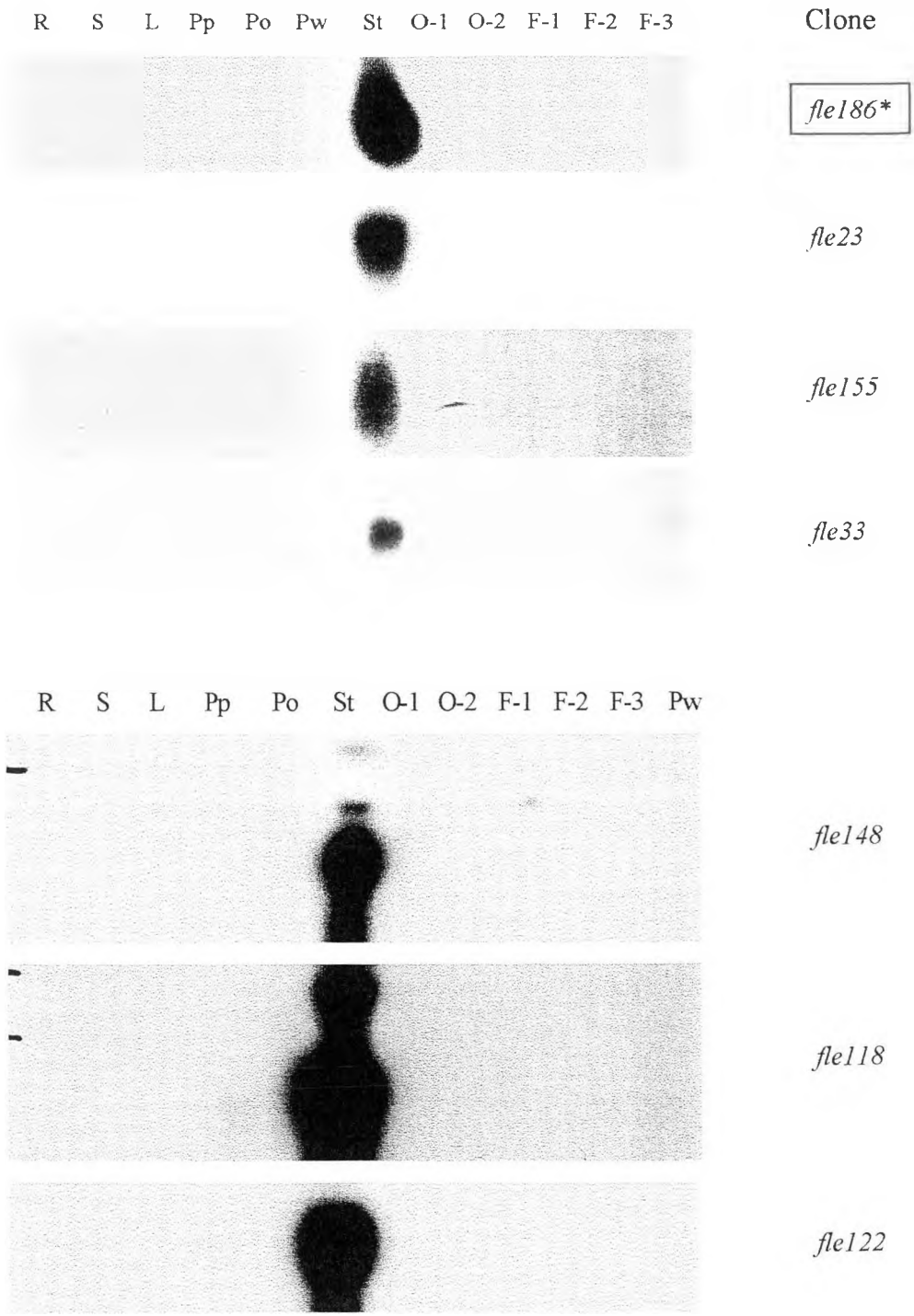
Northerns of RNA derived from tomato: Roots (R), stems (S), leaves (L), petals of open flowers (P), stamens (St), ovaries at pre-anthesis (O-1), ovaries of open flowers (O-2), fruits of 4-6mm in diameter (F-1), fruits of 8-10mm in diameter (F-2), fruits of 17-25mm in diameter (F-3), and mature green fruits (MG), probed with radiolabled DNA of the relevant clones.



* the boxed clone represent the gene which promoter is being analyzed

Figure 2. Expression patterns of different stamens specific cDNA clones

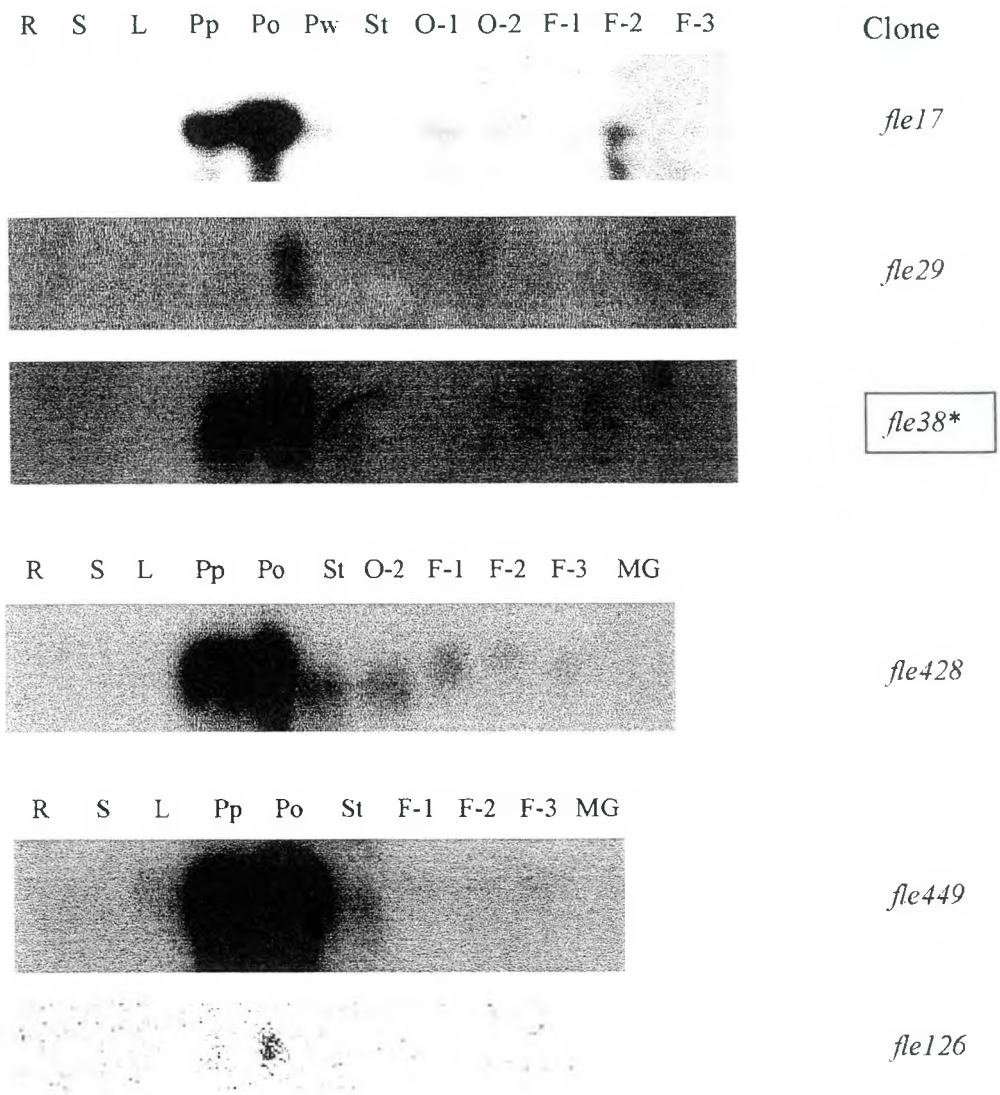
Northerns of RNA derived from tomato: Roots (R), stems (S), leaves (L), petals at pre-anthesis (Pp), petals of open flowers (Po), wilted petals (Pw), stamens (St), ovaries at pre-anthesis (O-1), ovaries of open flowers (O-2), fruits of 4-6mm in diameter (F-1), fruits of 8-10mm in diameter (F-2), fruits of 17-25mm in diameter (F-3), and mature green fruits (MG), probed with radiolabled DNA of the relevant clones.



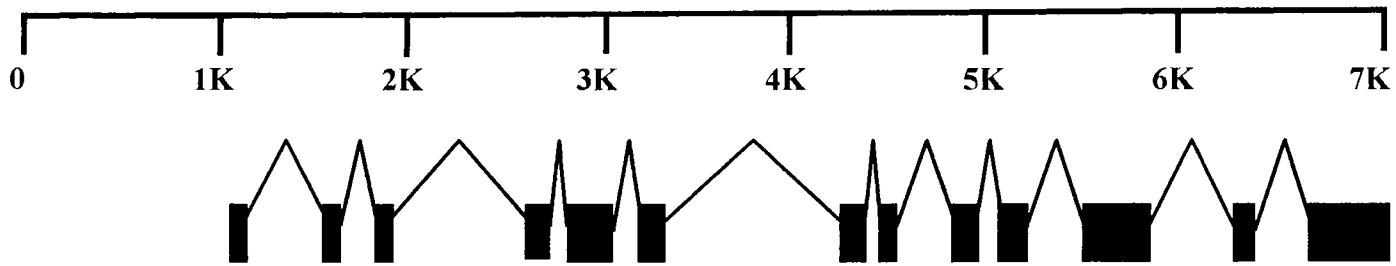
* the boxed clone represent the gene which promoter is being analyzed

Figure 3. Expression patterns of different petals expressed cDNA clones

Northerns of RNA derived from tomato: Roots (R), stems (S), leaves (L), petals at pre-anthesis (Pp), petals of open flowers (Po), wilted petals (Pw), stamens (St), ovaries at pre-anthesis (O-1), ovaries of open flowers (O-2), fruits of 4-6mm in diameter (F-1), fruits of 8-10mm in diameter (F-2), fruits of 17-25mm in diameter (F-3), and mature green fruits (MG), probed with radiolabled DNA of the relevant clones.



* the boxed clone represent the gene which promoter is being analyzed



Fiure 4. Alignment of cDNA and genomic sequence of petal expressed GDI fle38

Exons appear as boxes. The exons include all of the ORF and the 3' UTR.