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Regulation of S-Rnase gene expression in petunia hybrida

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Regulation of S-Rnase Gene Expression in *Petunia hybrida*

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ABSTRACT: Self-incompatibility is a cell-cell recognition system in higher plants that is based on the ability of the pistil to distinguish non-self pollen from self pollen. In *Petunia hybrida,* self-incompatibility is controlled by a single locus, the S-locus. The sequences governing S-RNase expression are not known and an understanding of this would enable us to better manipulate self-incompatibility response in flowers. This study attempted to correlate the degree of reporter gene activity with varying amount of 5' flanking sequence present on different gene constructs. Reporter gene activity was assayed by fluorometric measurements of GUS (β-glucuronidase) enzyme activity normalized to total soluble protein. Pistils were collected from transgenic plants with different gene constructs and protein extracts prepared. Extracts were used for protein and enzyme assays. Of the 34 plants assayed, only 5 showed GUS expression above background. Because of this low number of plants expressing GUS, we were unable to draw any conclusion about the effect of 5' flanking sequences on S-RNase expression.

Introduction

Various mechanisms in flowering plants have evolved to prevent the tendency of self-fertilization created by close proximity of male and female reproductive organs in a perfect flower. One such mechanism called self-incompatibility (SI), allows the pistil of a plant to reject self pollen or pollen from genetically related individuals, thus preventing inbreeding and promoting outcrosses. With this mechanism, in case of a self-pollination of a self-incompatible plant, pollen either fails to germinate or, if it does germinate, the pollen tubes develop abnormally. Self-fertilization is thereby averted. Genetic studies carried on earlier led to the identification of two different types of SI: gametophytic self-incompatibility (GSI) and sporophytic self-incompatibility (SSI). For GSI, the behavior of the pollen is determined by the genotype of the pollen grain itself, whereas for SSI, the SI behavior of the pollen is determined by the genotype of the parent plant. This difference most likely reflects the difference in the site of expression of the pollen S-allele (see Review Sims,1993).

In GSI, S-allele proteins are non-specific ribonucleases referred to as S-RNases. These proteins are required for self-incompatibility in styles. It has been seen that lowering the level of S-RNase mRNA has direct correlation to the loss of self-incompatibility in styles. The S-locus mRNA accumulates to high levels during the development of a flower, with the most pronounced increase occurring when the plant becomes self-incompatible from being self-compatible. Studies have shown that S-RNase protein is secreted into the inter-cellular spaces of the transmitting tract, where it accumulates to high levels (Sims,1993).

In order to analyze the sequences regulating the specific developmental expression of S-locus, Clark *et al* (1994) used microprojectile bombardment to introduce S-allele/β-glucuronidase (GUS) fusion genes into detached styles or intact pistils for transient expression assays. Various deletion clones having different amounts of S_1 5' flanking sequence were fused to the GUS reporter gene. They noted that plants with the different constructs all showed positive histochemical staining in styles and petals. Constructs having only 19bp of 5'flanking DNA, and lacking a TATA box as well as promoter-less GUS plasmids did not show positive histochemical staining.

The purpose of this paper was to find out (1) whether the S_1-GUS constructs were expressed in transgenic *Petunia hybrida*, (2) whether they were expressed in the same qualitative fashion as endogenous S-RNase and (3) whether there was variation in quantitative expression related to the gene construct (i.e. amount of 5'flanking sequence used).

Materials and Methods

Preparation for Assays:

Styles were collected from flowers of plants transformed with different constructs. Extracts were made by grinding the styles in liquid nitrogen and then adding the frozen powder to 2 ml of GUS extraction buffer (1<u>M</u> Na_2HPO_4, 1<u>M</u> NaH_2PO_4, 0.5<u>M</u> Na_2EDTA pH 8, 10% Triton X-100, 0.1% Na-N-lauroyl-sarcosine). The cell debris was pelleted in a microcentrifuge at maximum speed for ten minutes, the supernatant taken out and either used for the assays directly or frozen for use in future assays.

Protein Assay :

First a protein standard assay was done so that a standard curve could be determined. For that seven test tubes were set up with 0.1ml of diluted protein standard (Bio-Rad) with actually had 0μg, 13μg, 26μg, 52μg, 77μg, 103μg and 129μg of the protein standard. 5ml of diluted dye reagent was added to these tubes and they were let to stand for 15-30 minutes. After this, absorbance was read at A_{595} using a spectrophotometer and a standard curve drawn with the protein concentration on the x-axis and the absorbance along the y-axis.

For the actual protein assays, 100μl of extract was added to 5ml of diluted dye. If the absorbance reading was off scale, further dilutions were sometimes made with 50μl of the extract and 50μl of extraction buffer instead of the 100μl of the extract. The absorbance was then read and this value was used to find out the corresponding protein concentration from the standard curve.

Fluorometric Assays of GUS Activity:

250μl of extract was added to 250μl of pre-warmed assay buffer (2mM methlyumbelliferyl-β-D-glucuronide, MUG, in extraction buffer). 100μl of this solution was then added to 1.9ml of stop buffer (0.2M $NaCO_3$). The remaining assay solution was placed in a 37°C waterbath. 100μl aliquots were taken out at 30, 60 and 120 minutes after the initial extraction. Fluorescence was measured using a Hoeffer TKO fluorimeter with 500 fluorescent units (FU) equal to 50nM 4-methylumbelliferone (MU).

Calculations :

A graph was plotted with absorbance on the y-axis and time on the x-axis. The gradient of this graph was later used for the calculation of specific enzyme activity.

1. The corresponding concentration of protein ([prot]) was read off of a standard curve showing absorbance against [prot] in mg/ml.
2. This was then converted into prot in mg/ml.
3. The readings from the fluorometric analysis were plotted for each plant and the slope of this line gave us the fluorescence unit (FU)/min.

4. To calculate Specific Enzyme Activity (SA):

a) Conversion of FU/min to nM/min.

For standard (50n$\underline{M}$), FU = 500

In 2.0ml, 50 n$\underline{M}$ = 1×10^{-10} moles MU.

b) [(FU min^{-1}) X (2ml/0.1ml*) X (0.5ml/extract vol)]

TFU = FU X 20 X 2 = (40 X FU) FU $min^{-1}ml^{-1}$

or FU X 40 X 10 = (400 X FU)

note: extract volume is either 0.25ml or 0.1ml depending on the dilutions.

* or 0.05ml depending on dilutions.

c) $$\frac{TFU\ min^{-1}}{500} \times 1 \times 10^{-10} \text{ moles} = \frac{\text{moles MU}}{\text{min}}$$

$$= \frac{(40)(FU)}{500} \text{ or } \frac{(400)(FU)}{500} = [(0.08 X FU) or (0.8 X FU)(1 X 10^{-10})]$$

d) $$SA = \frac{\text{moles MU } min^{-1}\ ml^{-1}}{[prot]} = \text{moles MU } min^{-1} mg^{-1}$$

$$= \{(0.08 \times FU)/[prot] \text{ or } (0.8 \times FU)/[prot]\}(1 \times 10^{-10})$$

e) conversion of moles MU $min^{-1}mg^{-1}$ to nmoles MU

$min^{-1}mg^{-1}$ = SA/ 1×10^{-9}

or SA = {(0.08 X FU) X 0.1}/[prot] or {(0.8 X FU) X 0.1}/ [prot].

Example of Calculation:

For plant # 1-3,

prot abs = 0.54 = 30.93 µg/ml prot = 0.309 mg/ml prot

[prot] = 0.309

FU = 29.68

Therefore, SA=[(0.008 X 29.68)/0.309]=0.768 nmole MU $min^{-1}mg^{-1}$

Results

Deletion Constructs and Plant Transformation

In an attempt to specfically identify cis-acting regulatory sequences, a series of exonuclease-III deletion clones (Clark and Sims, 1994) containing variable amounts of 5'-flanking sequence, were subcloned into the transformation vector pGSC1700 (Cornellisen and Vandewiele, 1989). The deletion clones used in this study have the following amounts of 5'-flanking sequence: #1--- none (GUS cassette only), #2--- 8kb, #4 --- 1.9kb, #5 ---425 bp. These constructs were used for transformation of leaf disks of a *Petunia hybrida* S_1S_1 line, and plants regenerated (Ashraf and Sims, unpublished). Transformed plants were selected by regeneration on media containing kanamycin.

Flurometric Assays of β-glucuronidase (GUS) Activity in Transgenic Plants

Thirty four individual plants were assayed for GUS activity (see Methods). Of these, 1 was #1 (pUC-GUS), 7 were #2 (S_1-8kb-GUS), 14 were #4 (S_1-2kb-GUS) and 12 were #5 (S_1-450-GUS). Also, there were 4 non-transformed S_1S_1 which were used as controls. Out of all the plants assayed , only 5 plants showed expression greater than the background (Table 1). The result of the assays is given in Table 2.

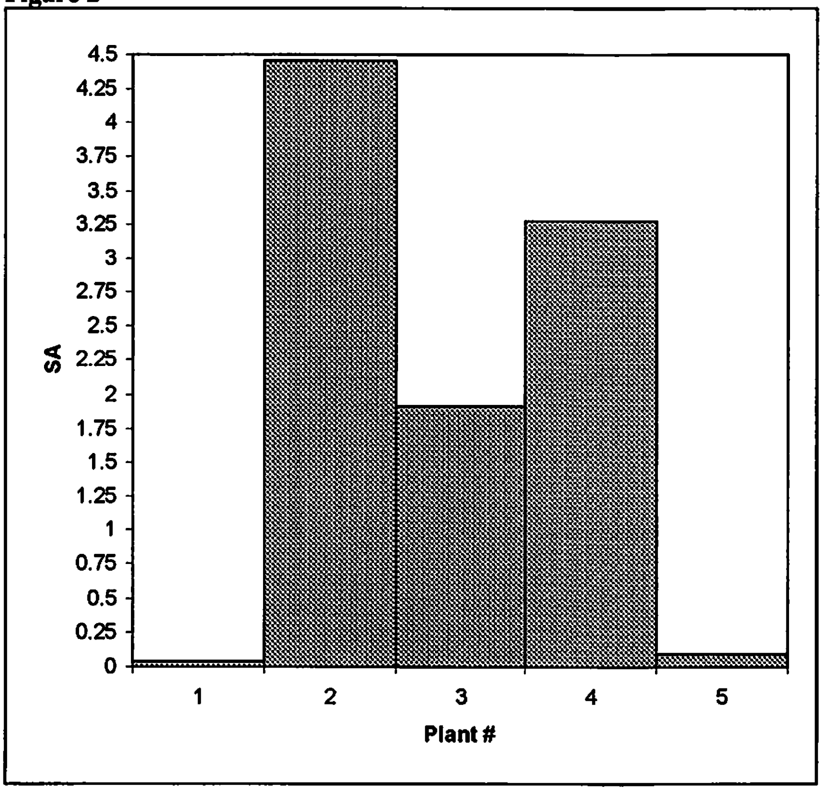
Calculations after data collection:

Plants showing greater expression than background:

Table 1

#	Plant #	Average SA(nM/min/mg)	Difference (AvgSA-x)
1	2-32	1.271	0.039
2	4-27	5.69	4.458
3	4-9	3.14	1.911
4	4-13	4.51	3.273
5	4-20	1.33	0.098

Figure 2



Mean of background (controls), x = 1.232

Table 2.

#	Plant	prot abs	[prot μg]	[prot mg/ml]		time					FU/min	nM/min/ml	nM/min/mg
						0	30	60	90	120			
241	1-3	0.54	30.93	0.309	FU	104	958	1921	nd	nd	29.68	0.237	0.768
					nM	10.4	95.8	192.1			2.97		
241'	1-3	0.55	31.54	0.315	FU	107	863	1706	nd	nd	26.65	0.213	0.677
					nM	10.7	86.3	170.6			2.67		
191	2-41	0.44	53.09	0.531	FU	87	585	1022	nd	1835	14.45	0.116	0.218
					nM	8.7	58.5	102.2		183.5	1.45		
191'	2-41	0.45	54.67	0.547	FU	80	515	859	nd	1600	12.53	0.100	0.183
					nM	8	51.5	85.9		160	1.25		
198	2-2	0.19	13.48	0.135	FU	62	345	610	nd	1114	8.73	0.070	0.517
					nM	6.2	34.5	61		111.4	0.87		
198'	2-2	0.19	13.48	0.135	FU	65	398	696	nd	1327	10.47	0.837	0.620
					nM	6.5	39.8	69.6		132.7	1.05		
214	2-16	0.35	19.41	0.194	FU	78	504	893	nd	1836	14.63	0.117	0.603
					nM	7.8	50.4	89.3		183.6	1.46		
214'	2-16	0.34	18.81	0.188	FU	81	498	902	nd	1788	14.22	0.114	0.605
					nM	8.1	49.8	90.2		178.8	1.42		
217	2-32	0.24	12.74	0.127	FU	67	648	1275	nd	nd	20.13	0.161	1.268
					nM	67	64.8	127.5			2.01		
217'	2-32	0.25	13.35	0.133	FU	74	672	1345	nd	nd	21.18	0.169	1.274
					nM	7.4	67.2	134.5			2.12		
221	2-38	0.33	18.20	0.182	FU	80	613	1191	nd	nd	18.52	0.148	0.814
					nM	8	61.3	119.1			1.85		
221'	2-38	0.34	18.81	0.188	FU	78	631	1214	nd	nd	18.93	0.151	0.805
					nM	7.8	63.1	121.4			1.89		
260	2-48	0.31	16.99	0.170	FU	88	550	1041	1584	nd	16.6	0.133	0.781
					nM	8.8	55	104.1	158.4		1.66		
260'	2-48	0.31	16.99	0.170	FU	92	600	1150	1616	nd	17.07	0.137	0.803
					nM	9.2	60	115	161.6		1.7		
289	2-19	0.38	21.23	0.212	FU	102	740	1432	nd	nd	22.17	0.177	0.837
					nM	10.2	74	143.2			2.21		
289'	2-19	0.39	21.84	0.218	FU	93	689	1319	1912	nd	20.29	0.162	0.745
					nM	9.3	68.9	131.9	191.2		2.03		
1	4-37	0.65	34.1	0.341	FU	73	149	449	nd	1020	8.26	0.066	0.194
					nM	7.3	14.9	44.9		102	0.83		
1'	4-37	0.66	34.7	0.347	FU	nd	nd	nd	nd	nd			
3	4-14	0.54	27.9	0.279	FU	87	575	1058	nd	nd	16.18	0.129	0.464
					nM	8.7	57.5	105.8			1.62		
3'	4-14	0.55	28.5	0.285	FU	96	626	976	nd	1947	15.19	0.122	0.426
					nM		62.6	97.6		194.7	1.51		

#	Plant	prot abs	[prot ug]	[prot mg/ml]		time					FU/min	nM/min/ml	nM/min/mg
						0	30	60	90	120			
*4	4-27	0.4	20	0.2	FU	172	644	1087	nd	nd	15.25	1.22	6.1
					nM	17.2	64.4	108.7			1.53		
*4'	4-27	0.41	20.6	0.206	FU	187	593	1002	nd	1820	13.61	1.090	5.28
					nM	18.7	59.3	100.2		182	1.36		
*6	4-9	1	53.8	0.538	FU	184	814	1407	nd	nd	21.22	1.698	3.15
					nM	18.4	81.4	140.7			2.12		
*6'	4-9	0.99	53.3	0.533	FU	193	829	1447	nd	nd	20.90	1.672	3. 137
					nM	19.3	82.9	144.7			2.09		
*7	4-13	0.43	21.7	0.217	FU	48	515	900	nd	1658	13.28	1.062	4.89
					nM	4.8	51.5	90		165.8	1.33		
*7'	4-13	0.45	22.9	0.229	FU	59	443	808	nd	1476	11.76	0.981	4.12
					nM	5.9	44.3	80.8		147.6	1.18		
12	4-17	0.49	25.78	0.258	FU	250	748	1350	nd	nd	18.33	0.147	0.56
					nM	25	74.8	135			1.83		
12'	4-17	0.48	25.22	0.252	FU	250	797	1505	nd	nd	20.91	0.167	0.66
					nM	25	79.7	150.5			2.09		
13	4-15	0.28	14.20	0.142	FU	257	606	1070	nd	1566	11.00	0.088	0.62
					nM	25.7	60.6	107		156.6	1.10		
13'	4-15	0.29	14.75	0.148	FU	262	620	1112	nd	1575	11.04	0.088	0.59
					nM	26.2	62	111.2		157.5	1.10		
*15	4-20	0.54	28.53	0.285	FU	47	193	319	471	619	4.74	0.380	1.33
					nM	4.7	19.3	31.9	47.1	61.9	0.47		
18	4-26	0.53	27.98	0.280	FU	73	674	1320	nd	nd	20.78	0.166	0.59
					nM	7.3	67.4	132			2.08		
18'	4-26	0.54	28.53	0.285	FU	73	701	1320	nd	nd	20.78	0.166	0.583
					nM	7.3	70.1	132			2.08		
19	4-31	0.56	29.63	0.296	FU	75	679	1301	1987	nd	20.19	0.161	0.55
					nM	7.5	67.9	130.1	198.7		2.02		
19'	4-31	0.56	29.63	0.296	FU	75	676	1283	1904	nd	20.31	0.162	0.55
					nM	7.5	67.6	128.3	190.4		2.03		
43	4-18	0.44	53.09	0.531	FU	63	426	853	nd	1600	12.89	0.103	0.19
					nM	6.3	42.6	85.3		160	1.29		
43'	4-18	0.44	53.09	0.531	FU	67	476	966	nd	1815	14.67	0.117	0.22
					nM	6.7	47.6	96.6		181.5	1.47		
69	4-19	0.34	37.25	0.373	FU	86	587	1192	nd	nd	18.43	0.147	0.39
					nM	8.6	58.7	119.2			1.84		
69'	4-19	0.32	34.08	0.341	FU	84	609	1020	nd	nd	15.6	0.124	0.37
					nM	8.4	60.9	102			1.56		
73	4-37	0.36	40.41	0.404	FU	79	606	1135	nd	nd	17.60	0.141	0.35
					nM	7.9	60.6	113.5			1.76		

#	Plant	prot abs	[prot ug]	[prot mg/ml]		time					FU/min	nM/min/ml	nM/min/mg
						0	30	60	90	120			
73'	4-37	0.36	40.41	0.404	FU	80	623	1177	nd	nd	18.28	0.146	0.36
					nM	8	62.3	117.7			1.83		
233	4-40	0.24	12.74	0.127	FU	65	536	1045	nd	1986	16.05	0.128	1.01
					nM	6.5	53.6	104.5		198.6	1.61		
233'	4-40	0.24	12.74	0.127	FU	65	529	1033	nd	1901	15.33	0.123	0.97
					nM	6.5	52.9	103.3		190.1	1.53		
8	5-10	0.47	24	0.24	FU	535	991	1435	nd	1789	10.30	0.082	0.34
					nM	53.5	99.1	143.5		178.9	1.03		
8'	5-10	0.48	24.5	0.245	FU	541	1005	1478	nd	1987	11.96	0.096	0.39
					nM	54.1	100.5	147.8		198.7	1.20		
9	5-24	0.37	19.16	0.192	FU	235	546	914	nd	1575	11.25	0.09	0.47
					nM	23.5	54.6	91.4		157.5	1.12		
9'	5-24	0.37	19.16	0.192	FU	237	629	1020	nd	1778	12.83	0.103	0.53
					nM	23.7	62.9	102		177.8	1.28		
10	5-20	0.28	14.20	0.142	FU	92	538	980	nd	1919	15.23	0.122	0.86
					nM	9.2	53.8	98		191.9	1.52		
10'	5-20	0.29	14.75	0.148	FU	90	507	970	nd	1871	14.91	0.119	0.81
					nM	9	50.7	97		187.1	1.49		
20	5-3	0.54	28.53	0.285	FU	63	433	823	nd	1555	12.45	0.100	0.35
					nM	6.3	43.3	82.3		155.5	1.25		
20'	5-3	0.54	28.53	0.285	FU	64	448	811	nd	1496	11.95	0.096	0.33
					nM	6.4	44.8	81.1		149.6	1.20		
29	5-9	0.4	20.82	0.208	FU	104	659	1207	nd	nd	18.38	0.147	0.71
					nM	10.4	65.9	120.7			1.84		
29'	5-9	0.4	20.82	0.208	FU	104	646	1214	nd	nd	18.50	0.148	0.71
					nM	10.4	64.6	121.4			1.85		
30	5-21	0.42	21.92	0.219	FU	100	577	1059	nd	nd	15.98	0.128	0.58
					nM	10	57.7	105.9			1.60		
30'	5-21	0.4	20.82	0.208	FU	104	590	1137	nd	nd	17.22	0.138	0.66
					nM	10.4	59	113.7			1.72		
31	5-18	0.39	20.27	0.203	FU	99	683	1219	nd	nd	18.67	0.149	0.74
					nM	9.9	68.3	121.9			1.87		
31'	5-18	0.39	20.26	0.207	FU	100	643	1246	nd	nd	19.10	0.153	0.74
					nM	10	64.3	124.6			1.91		
36	5-32	0.28	14.20	0.142	FU	98	473	879	nd	1458	11.33	0.091	0.64
					nM	9.8	47.3	87.9		145.8	1.13		
36'	5-32	0.28	14.20	0.142	FU	96	473	869	nd	1636	12.85	0.10	0.72
					nM	9.6	47.3	86.9		163.6	1.29		
37	5-30	0.43	22.47	0.225	FU	90	653	1190	nd	nd	18.33	0.147	0.65
					nM	9	65.3	119			1.83		

#	Plant	prot abs	[prot ug]	[prot mg/ml]		time					FU/min	nM/min/ml	nM/min/mg
						0	30	60	90	120			
37'	5-30	0.44	23.02	0.230	FU	91	618	1093	nd	nd	16.70	0.134	0.58
					nM	9.1	61.8	109.3			1.67		
38	5-4	0.56	72.10	0.721	FU	93	453	908	nd	1766	14.09	0.113	0.16
					nM	9.3	45.3	90.8		176.6	1.41		
38'	5-4	0.56	72.10	0.721	FU	94	471	925	nd	1680	13.31	0.106	0.148
					nM	9.4	47.1	92.5		168	1.33		
56	5-22	0.45	54.67	0.547	FU	80	525	1071	nd	1812	14.52	0.116	0.21
					nM	8	52.5	107.1		181.2	1.45		
58	5-15	0.44	53.09	0.531	FU	73	415	793	nd	1540	12.28	0.098	0.18
					nM	7.3	41.5	79.3		154	1.23		
58'	5-15	0.45	54.67	0.547	FU	73	407	804	nd	1500	11.97	0.096	0.18
					nM	7.3	40.7	80.4		150	1.20		
*I	97V(90)FS2	0.54	68.93	0.689	FU	95	366	575	nd	980	7.27	0.582	0.844
					nM	9.5	36.6	57.5		98	0.73		
*I'	97V(90)FS2	0.52	65.76	0.658	FU	94	366	570	nd	1020	7.61	0.608	0.925
					nM	9.4	36.6	57		102	0.76		
*II	97V(90)FS2	0.3	30.91	0.309	FU	87	330	500	nd	870	6.41	0.513	1.659
					nM	8.7	33	50		87	0.64		
*II'	97V(90)FS2	0.28	27.74	0.277	FU	87	319	493	nd	832	6.12	0.480	1.767
					nM	8.7	31.9	49.3		83.2	0.61		
*III	97V(90)FS2	0.43	51.50	0.515	FU	95	338	530	nd	897	6.59	0.527	1.02
					nM	9.5	33.8	53		89.7	0.66		
*III'	97V(90)FS2	0.41	48.33	0.483	FU	95	319	503	nd	857	6.28	0.502	1.04
					nM	9.5	31.9	50.3		85.7	0.63		
*IV	97V(90)FS2	0.28	27.74	0.277	FU	84	259	395	nd	660	4.73	0.378	1.37
					nM	8.4	25.9	39.5		66	0.47		

Discussion

Out of all the plants (34) that were used for the fluorometric assays only five (14.7%) of them showed GUS expression greater than the background. Of these plants, one was plant 2-32 with the construct S_1-8kb-GUS and the other four were plants 4-27, 4-9, 4-13 and 4-20 with the constructs S_1-2kb-GUS.

No conclusions can be drawn on the effect of 5' flanking sequence amount on gene expression based on these results. The reasons why so few plants showed GUS expression could be either of the following: (1) The plants were not transformed in the first place. This is unlikely since these plants were Kan^R when tested, and therefore should have the transformed construct.

(2)Alternatively, only a few transgenic plants showed high levels of GUS expression, probably due to "position effects". Earlier experiments (see Sims *et al* 1993), had also given mixed results. For example, Murfett *et al* (1995), had tested the effects of S_2, S_6 and S_{A2} promoters from *Nicotiana alata* in several hosts. Expression was only observed with the S6 promoter in *N. alata*, and in that case the level of expression was estimated to be 300-fold below that of endogenous S6 expression. Also, in work done by Lee *et al* (1994), only about 3% of the total transformants assayed gave levels of expression comparable to endogenous S-RNase expression.

The number of plants that I had screened was probably not sufficient to draw any definite conclusion. Work with transgenic plants have shown that a large number of plants have to be screened before any lines expressing transgenes can be obtained.

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