

(P6013. 1991)

최        중  
연구보고서

딸기바이러스병 진단기술 개발과 이용

Development of Techniques for Diagnosing  
Viral Diseases to  
Strawberry, *Fragaria grandiflora* Ehrn.

전 남 대 학 교

농림부행정자료실



0007695

농 립 부

1999

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I.

II.

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III.

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- 가 가 4

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- (Pol yconal ant i body)  
(Mnocl onal ant i body)

- RT-PCR

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IV.

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1	-----	5
1	-----	5
1.	-----	5
2.	-----	6
2	-----	8
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1.	-----	8
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3	-----	10
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2.	-----	14
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4.	-----	24
3	-----	25
1	-----	25
2	-----	27
1.	-----	27
3	-----	38
1.	-----	38
2.	-----	39
4	-----	63
1	-----	63
2	-----	64
1.	-----	64
3	-----	64
1.	-----	64
2.	-----	66
3.	ELISA -----	67
	-----	72

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(TEM)

acryl ami de

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core RNA

cDNA

RT-PCR

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	0	0	0	0
	0		1	
	6	1,650	0	
	7	105	0	
	12	126	30	449
	135	592	9	167
	90	1,021	56	879
	82	976	113	1,603
*	596	6,530	1,707	39,727
	49	716	487	7,446
*	456	5,349	1,265	35,001
	91	1,030	462	10,761
*	152	2,452	1,565	33,842
	217	2,604	9	131

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( g kg).

1g

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		가			(isolator)		
		'97	'98	'99	'97	'98	'99
	( )	51	32	11	111	87	12
		38	27	15	13	12	10
		8	5		15	17	
		7	13		12	8	3
		7			12	13	
		2		1	2		
	( )	17			14		
		7			11		
		9			3		
	( )	32	18	8	113	23	
		12	12	10	16	15	
		21	10	3	11	8	
	( )	6			2		
			8			7	
	( )			30		28	

221 가

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335

i sol at e

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219 가

12 가

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	41,	2	2
	12,	1	
	42,	5	
	7,	1	
	3,	1	
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	28,	2	
	3,	1	
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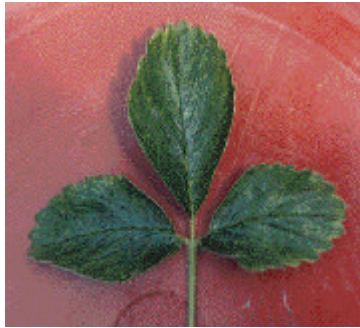
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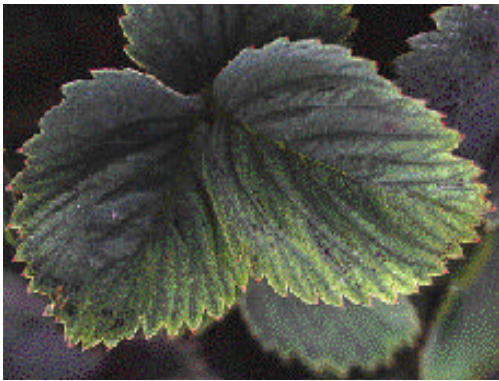




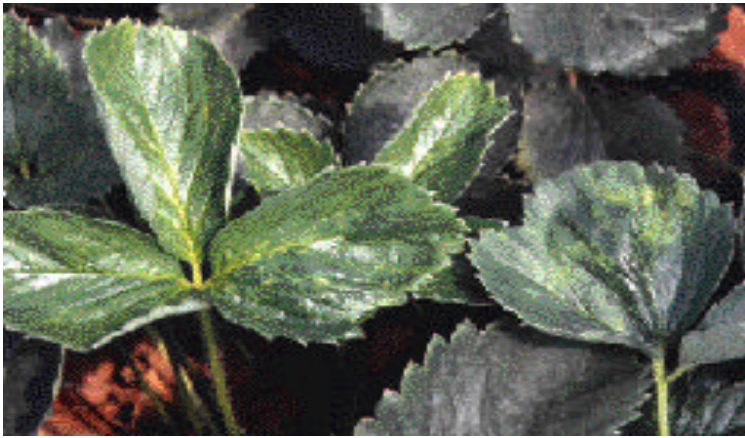
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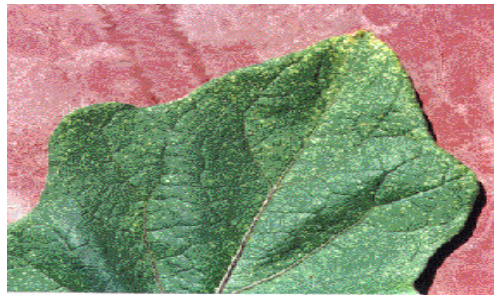


< 7> 2  
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4

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6

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95

가 가

가

3

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作物	同定 類數	研究水準
	3	同定, 診斷, 生態, 防除
,	3	同定, 生態
	14	同定, 診斷, 傳染
	35	同定, 診斷, 防除
	8	同定, 診斷, 生態, 防除
	5	同定, 診斷, 生態,
	3	同定

0

- :

- :

- : , ,

3 ( , 가 , )  
가 conjugate

( ) fragment . 가 가  
( ) 가

, 가

가

가  
가

가

가

가

가

가

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DECORATION

2가

가

가

260nm

280nm

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cesi um densi ty gradi ent

cent ri fugat i on

2

1 . ,

가.

SDS- PAGE .

food mi xer pol ytr on

pol yet hyl ene glycol

Tri s

pH

pH 6.5 .

4

가

w v 4-5

chl or of or

m 2

but anol .

4C .

<

>

Grind leaves in 0.5M borate buffer, pH 7.5 contained

0.1% thioglycolic acid

filtration through muslin

retain filtrate

discard residue

centrifuge at 5,000rpm for 30min

discard residue

---

Add 8.5% butanol to filtrate  
and stir for 45min

or

Add PEG 6000 to  
supernatant (10%)  
and shake for 30min

Centrifuge at 5000 rpm for 30min  
: retain supernatant

Centrifuge at 8000 rpm  
: discard supernatant

Centrifuge at 75000 g for 60min,  
suspend pellet in 0.0M borate buffer  
contained 2% triton-X100  
Leave overnight

Suspend pellet in 0.05M  
borate buffer

Centrifuge resuspended pellet  
at 8000rpm for 20min  
: discard pellet

Centrifuge at 45000 rpm  
at 150min  
: discard supernatant

Resuspended pellet  
: retain supernatant,  
Centrifuge 5000g for 20min

Chromatography or  
Gradient centrifugation

- Size exclusion column chromatography

Sepharose CL-6B

glass bead gel filtration (20 X 1200mm) flow rate : 1ml/  
min

- Affinity column chromatography

cellulose sulfate 가 sulf

ate 5% 가 1 X 3cm small column column buf

fer  
: binding - 0.02M phosphate buffer, pH 7.2  
: elution - 0.02M phosphate with 0.5M NaCl buffer

- Ultracentrifugation

○ isopycnic centrifugation - 44000rpm 5

○ gradient (sucrose) - 0.5ml 40% 0.6ml 36% 1ml 35% 1ml 34%

1ml 32% (w/w) CsCl , 0.02M phosphate buffer

○ centrifugation - 44000rpm 5

gel filtration

1kg

3kg

Polyclonal

5 Newzealand white 가 200ug  
Freund's complete adjuvant 1ml 가 50spot

2 50ug 3 가  
5 10 - 20ml 가 50- 100ml

1 4500rpm 20  
-20C Ri vanol IgG

가

500ml  
protein-A chromatography

-20C

30% glyc

erol -20C

1)

- Mouse

virus

200µg/ 100µl(0.9% saline

+ Freund's complete adjuvant, FCA)

8

Balb/C 5

2-3

3 50 $\mu$ g 가 . 8  
가

- *In vitro* immunization

3 - 4

가 가 . 2 3 - 4  $\mu$ g

2)

koehler & Milstein  
가 가 Balb/C (1.5  
X 10<sup>8</sup>) myeloma (1.5X10<sup>7</sup>) 50% polyethylene glycol (PEG) 1ml  
hybridoma 10% fetal calf serum + RPM1640 2  
PEG 가  
96well plate .

3) hybridoma

hybridoma cell 20% FCS- RPM 1640- HAT(hypoxanthine, aminopterin, thymidine) 가 96well plate  
HAT 가 HT 10%FCS- RPM 1640  
2 hybr idoma  
well plate 1/3 24well plate 2  
가 25cm<sup>2</sup> flask .  
MEM Na-  
gentamycin, penicillin, streptomycin .

4) Hybridoma 가

IgG antibody 가 . 가 IgG (anti mouse  
capture antibody(cAb) anti - mouse IgG



- alkaline phosphatase

virus coating

5) Ascitic fluid

Balb/C 1 0.5ml pristane 가  
hybridoma 1 1 hybridoma  
1 |  
가 protein A-Sepharose 4B column chromatography  
70

6) ELISA

- IgG coating

polyclonal monoclonal antibody  
polystyrene coating  
가 IgG  
capture antibody (cAb) BSA  
Balb/ protein A-Sepharose 4B IgG  
IgG 가 anti-mouse antibody  
IgG cAb cAb(200µg/well/ 100µl/ 50mM  
NaHCO<sub>3</sub>, pH 8.6) 96well/plate 가 4 16 coating  
coating 1% BSA/ 100µl / 50mM TBS 가 2 가

- conjugate

ELISA biotin  
conjugate 가 peroxidase alkaline phosphatase  
- phatase EDC glutaraldehyde  
30% glycerol 가 -20C

ELISA protocol

1. coat polystyrene microtiter plates
2. Decant thoroughly, add 0.3ml of dilute mAb and incubate for 3hrs, at 30 .
3. Decant and rinse twice with tap water
4. Add, to each cup, 0.05ml TBS, 0.1 of virus standard sample in TBS.
5. Incubate plates for 60min at 40 .
6. Add 0.05ml of dilute enzyme tracer, mix and incubate for 1-3hr. at 4 .
7. Decant and rinse twice with TBS.
8. Add 0.2ml of a fresh solution of 1ml p-nitrophenyl phosphate in 1ml of 50mM glycine buffer, pH 6.
9. Stop reaction with 0.05ml of 5MKCH
10. read absorbances at 405nm

ngstate      uranyl acetate      grid      phosphot u  
negative staining

PCR

1) - conjugate

conjugate      10mg horadi sh peroxi dase      1% gl ut ar al dehyde 0.2ml      가  
18      Sephadex G-25      gl ut ar al dehyde

brown 1ml volume  
 0.9% NaCl IgG (1ml) 가 Carbonate-bi car  
 bonate (0.5M pH 7.5) 0.2ml 가 4, 24 . phos  
 phate-buffered saline (pH 7.5, PBS )  
 가 4, 30 4000g X 20  
 . PBS glycerol 가 -2  
 0 . Alkaline phosphate co  
 njugation .

2) biotinylation

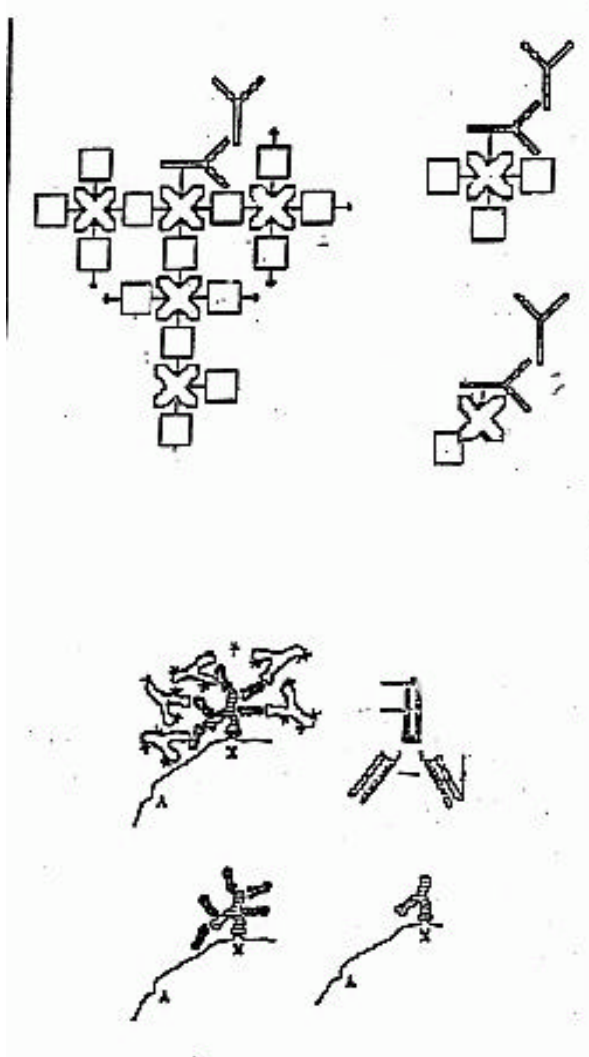
IgG bi carbonate ddydor Sephadex G-25  
 1mg/ml 75 $\mu$ l biotin (1mg biotinylation in 1ml DMS  
 O) 가 4 . 0.1M Tris-HCl (pH  
 7.4) 4 .

3) Fab, Fc

IgG pepsin, papain . (Pierce Kit )  
 fragment

FPLC  
 gel filtration column (30X1cm MCO: 300,000 )

< streptavidin-biotin conjugate >



streptavidin-biotin

conjugate

1                      1                      가                      -                      가                      가

streptavidin                      4x4                      가                      가



kit

가

가

conjugate,

buffer

가

biotin-avidin

I

gG

1). cDNA

phenol chloroform

ethanol

RNA

Poly Atract (Promega)

mRN

A

260nm

cDNA

first strand

superscript II (BRL)

second strand

Stratagene

Lambda-ZAP-cDNA synthesis kit

cDNA 5'

Eco RI, 3' poly(A)

Xho I

G

igapack II gold packaging extract (Stratagene)

*in vitro* packing

2).



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DECORATION

2가

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260nm

280nm

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column

cesium density gradient

centrifugation

2.



chromatography

Ani con

sul fate

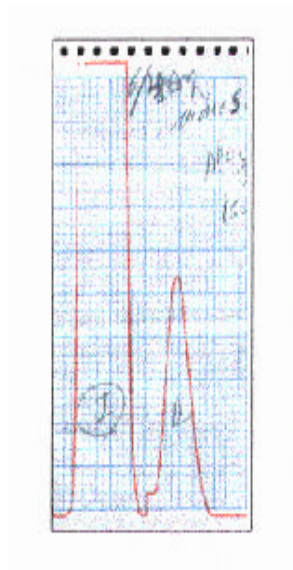
가 colum

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cellulose

가

가



< 12 >

acetate buffer

NaCl

sucrose gradient

가

가

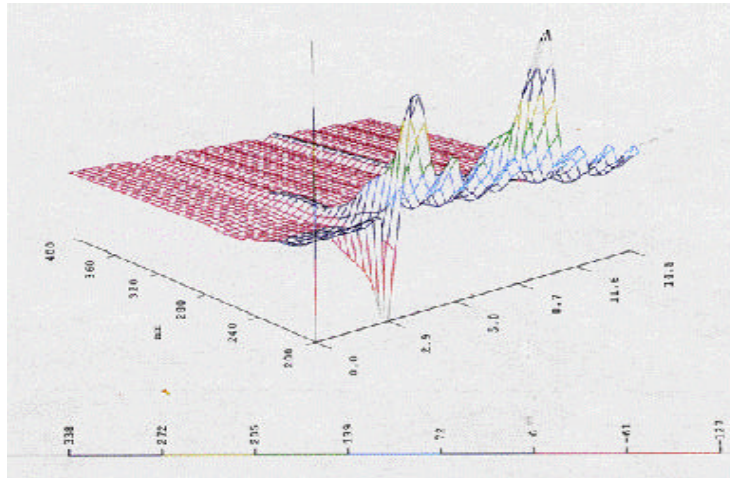
column chromatography

가

exclusion limit가

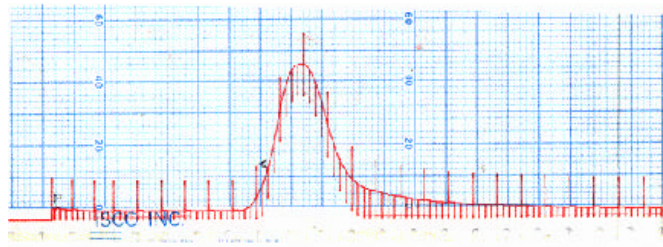
Sepharose 2B chromatography

가



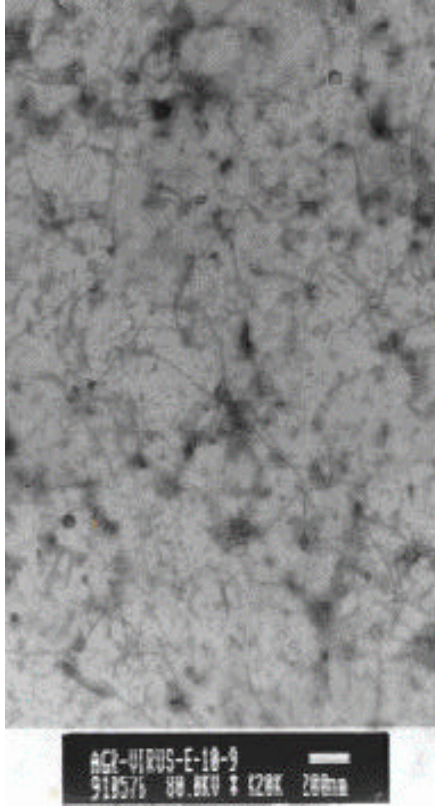
< . 13 >

가



< 14 > Sepharose 2B chromatogram ( 20 X 1200mm column )

가



( SV-7 )

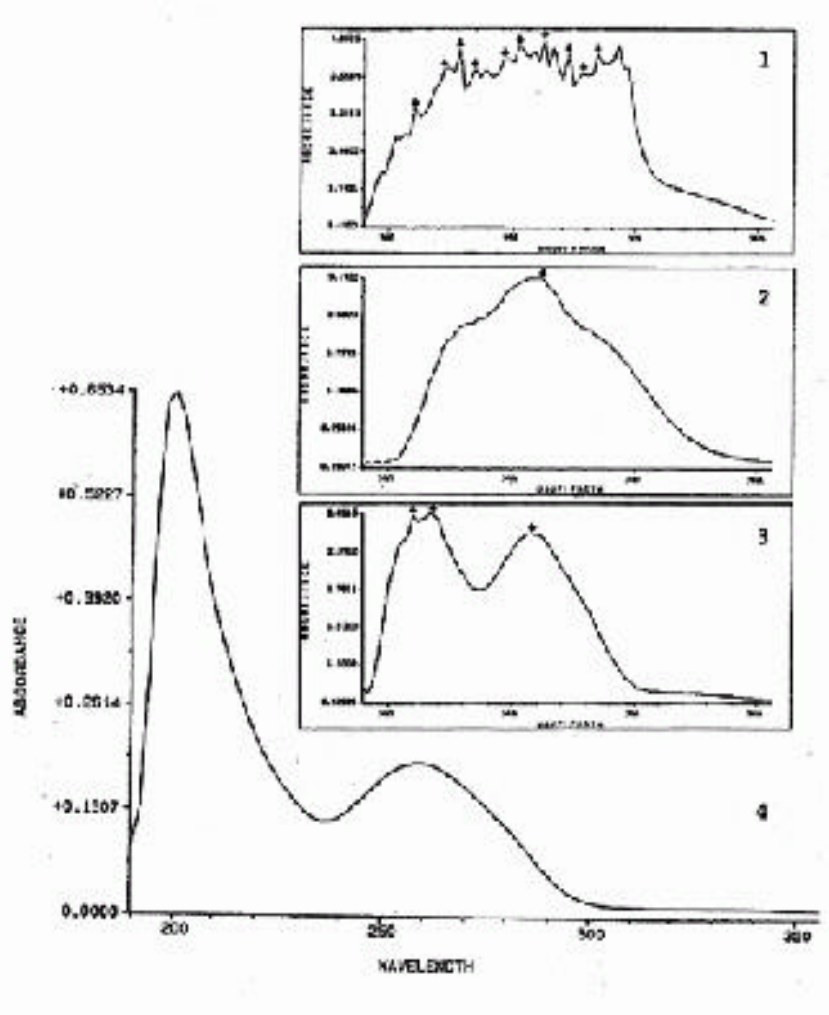
< 15 >

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I: , II : , III : sucrose gradient  
 IV: column cesium

isolates 가 1 20 2 10

가

< 5 > 30 isolates

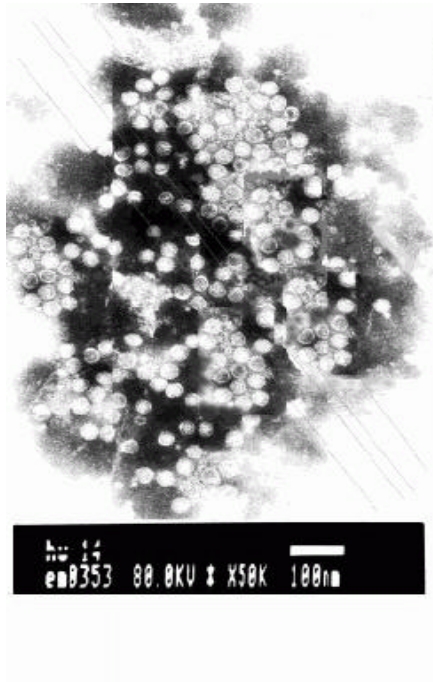
Sample NO	Isolates		Symptom expression	Virus		
SV-1	SJ-10-2	leaf				
SV-2	SJ-10-3			-	,,	,,
SV-3	SJ-10-8				,,	
SV-4	SJ-10-22		,		,,	,,
SV-5	SJ-16-5	fruit			,,	,,
SV-6	SJ-16-7	leaf			,,	
SV-7	SC-3-11					,,
SV-8	SC-5-8			-	,,	
SV-9	SC-5-16		,	-	,,	
SV-10	SC-7-3				,,	,,
SV-11	SC-9-11					,,
SV-12	SC-10-5					
SV-13	SJ-12-3		,	-	,,	,,
SV-14	SJ-12-7				,,	
SV-15	SJ-12-6		chlorosis wild twist curling		,,	,, ,,

Sample NO	Isolates		Symptom expression	Virus			
SV-16	SJ-13-2	leaf				,,	
SV-17	SB-1-3			-		,,	
SV-18	SB-2-6		curling	-	,,		
SV-19	SK-1-4			-	,,	,,	
SV-20	SK-1-7				,,	,,	
SV-21	SJ-13-2			,	,		
SV-22	SJ-12-4				-	,,	,,
SV-23	SJ-11-8		chlorosis		-	,,	
SV-24	SJ-8-12		wild twist			,,	,,
SV-25	SJ-12-5		curling				,,
SV-26	SJ-19-7						
SV-27	SC-13-11				-	,,	,,
SV-28	SC-15-8		curling			,,	
SV-29	SC-15-16					,,	
SV-30	SC-17-3						,,

가  
4가

가 가

### Virus I



1. ; SV-11

2.

;

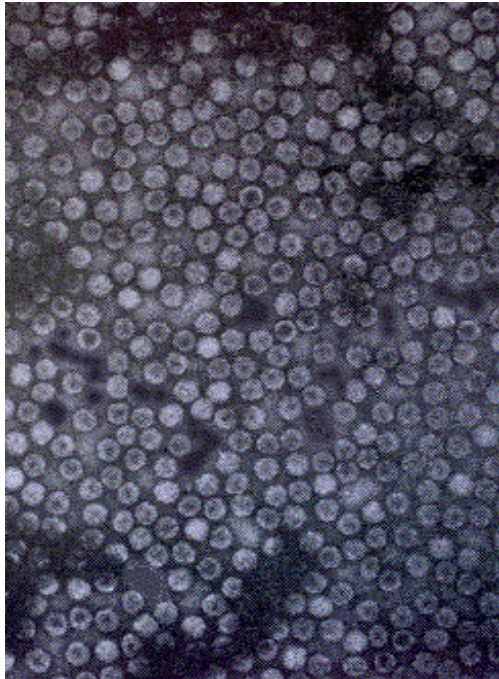
; 35 nm

3. ;

< 17 >



Virus II



1. ; SV- 20

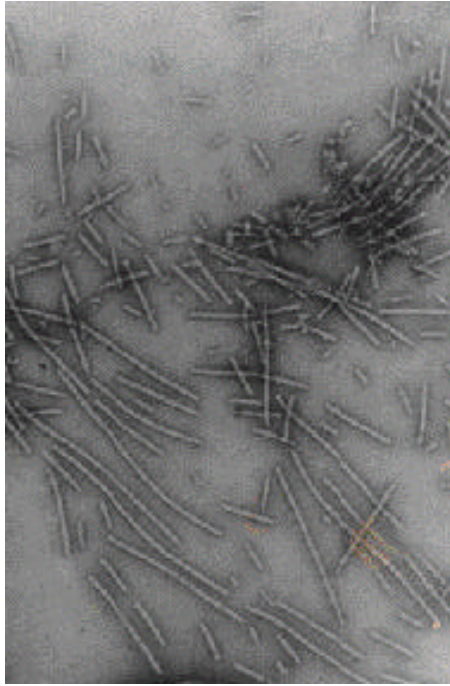
2.

; 30 nm

3. ;

< 18>

### Mrus III



1. ; SV- 4

2.

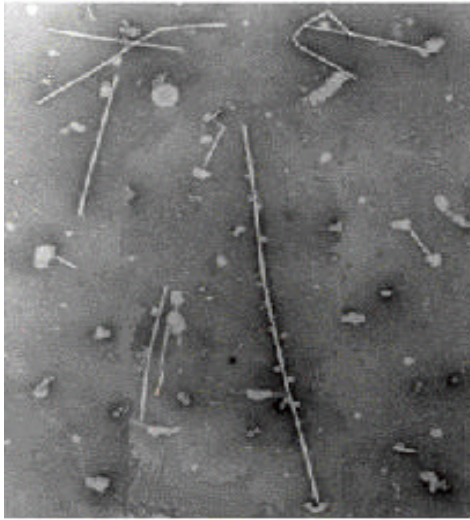
;

; 550nm

3. ;

< 19>

## Virus IV



1. ; SV- 15

2.

;

; 750 nm

;

3. ;

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	Rabbit	IgG ng / ml antiserum	50% binding
SV - 4	I	6	1: 500
	II	4	1: 1250
SV - 7	I	12	1: 2230
	II	11	1: 1500
SV - 11	I	8	1: 850
	II	8	1: 2200
SV - 15	I	16	1: 5000
	II	14	1: 3500
SV - 20	I	10	1: 1500
	II	11	1: 750

polyclonal

가

hybridoma cell

가

< 7 >

(monoclonal antibodies)

가

virus	Description	ml ascitic fluids / head (average)	IG isotype	50% binding
SV - 4	SWL-4-113	8	IgG <sub>a</sub>	1 : 12,500
	SWL-4-278	11	IgG <sub>a</sub>	1 : 22,500
	SWL-4-213	12	IgG <sub>2b</sub>	1 : 2,800
SV - 7	SWL-7-132	7	IgG <sub>a</sub>	1 : 22,000
	SWL-7-129	7.5		
	SWL-7-343	9		
SV - 15	SWL-11-213	10	IgG <sub>a</sub>	1 : 8,500
	SWL-11-217	11	IgG <sub>a</sub>	1 : 110,000
	SWL-11-326	9	IgG <sub>2b</sub>	1 : 32,500
SV - 20	SWL-20-147	12	IgG <sub>a</sub>	1 : 42,500
	SWL-20-237	9.5	IgG <sub>a</sub>	1 : 22,000
	SWL-20-281	11	IgG <sub>2b</sub>	1 : 32,000

4) 2 ( )  
*Fragaria vesca* *Fragaria virginiana* micro  
syringe  
가

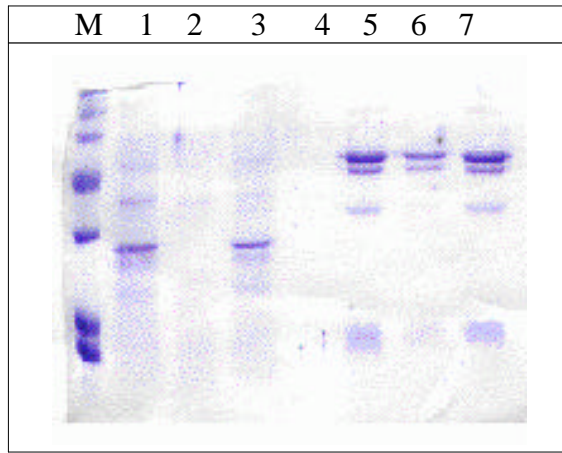


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5)-

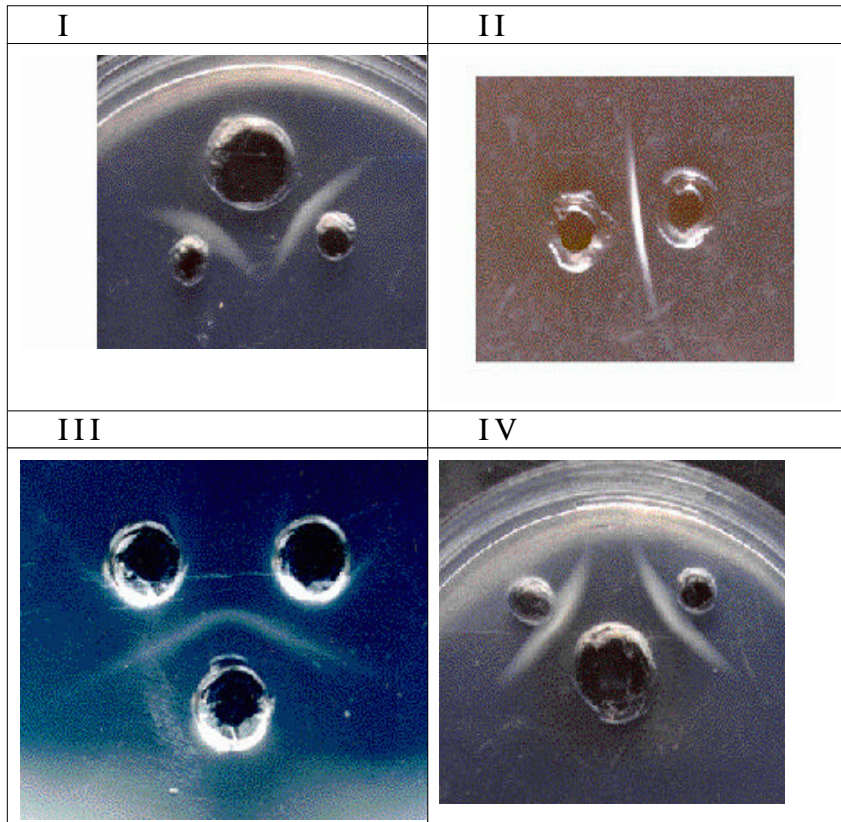
( 0.125M Tris-HCl /  
50% , 10% SDS / 1- % 2- mercapto ethanol / 0.005% bromophenol blue, pH  
6.8) 100C 2-3 가  
12% SDS- PAGE 90 15  
100 45 0.1% CBBR  
7.5% acetic acid, 5% methanol



< 22> ( ) NC filter  
blotting 가

M; maker, 1 - 7

6)



< >

Central well ; virus

Side wells ; monoclonal antibody

well  
 가 37C i ncubat or 12  
 가 . 가 가 .  
 가 가 가가  
 ELISA  
 가 . well  
 cell sap 가 가  
 가 .

7)

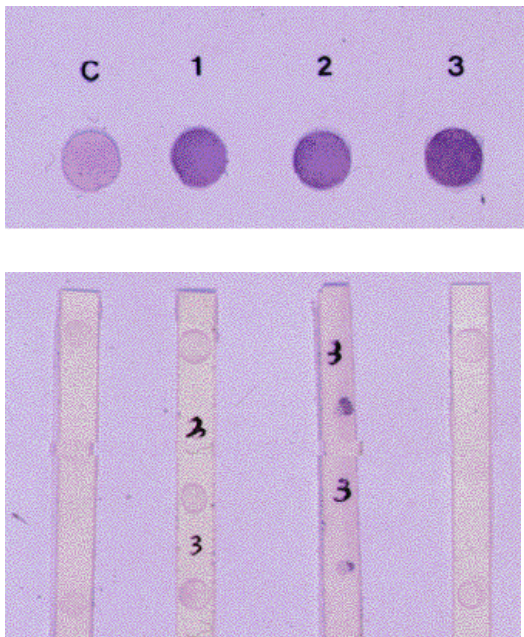
membrane filter  
 가 biotin  
 conj ugat e . - conj ugat e ELISA  
 가 . 가  
 .

< 8 > - conj ugat e

	Antigen (virus)	Monoclonal antibody	Enzyme	Coupling method
I	SV - 4	SWL-4-113 SWL-4-278 SWL-4-213	Peroxi dase,	
II	SV - 7	SWL-7-132 SWL-7-129 SWL-7-343	Al kal i ne phosphat ase	G ut al dehyde or NH <sub>2</sub> -LC- Bi ot i n
III	SV - 11	SWL-11-213 SWL-11-217 SWL-11-326	& Bi ot i n	
IV	SV - 20	SWL-20-147 SWL-20-237 SWL-20-281		

- conjugate ELISA 가 가  
 . 가  
 . ELISA cAB -  
 virus - - - conjugate .  
 .  
 conjugate 가 .  
 conjugate kit .

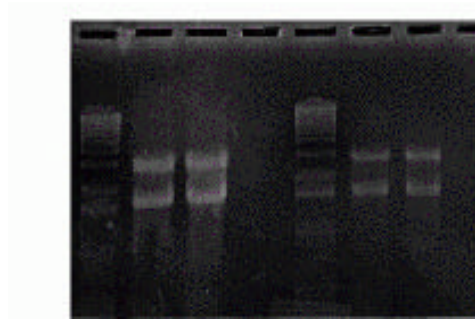
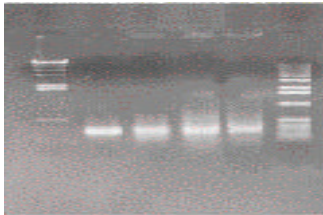
8) Dot blot



< 25> Dot blot . 가 가  
 30 가 3 - conjugate .  
 가 5

9)-

RNA  
cDNA  
RT-PCR  
cDNA  
PCR  
가 SV-4 SV-7  
protein-A Sepharose  
revers-transcriptase 1.25unit  
Taq polymerase, random primer F, R 1ul buffer 5ul 가 95C 30  
55C 30 72C 30 35cycle  
agrose gel



< 26> RNA RT-PCR

- cDNA

```

                                T:SK
CGACGACCGA  CGACTCAGCT  ACCTCACCGA  GTTCACCACC  GGACACCTCC
TTGGCGAATC  ACTATGGCAC  CTCCTCCCTG  AAACCCATGT  TCAGTATCAG
TCAGCCTGCT  TTGACTTCTT  CTGCAGGCGG  TGCCCAAGCA  ACGAGAAAAT
GCTCCTCGAC  GACTCCACAC  TCGCACTCAG  CCTCCTCGAA  CGAATCACTT
CTTCGCGGAG  GTGCCACAGC  GTTTCCACCC  TCACCACCTT  CTACCGTCAT
GCATCTCTGG  AATCACTCTG  GGTCACTATC  CATCCCACCT  TGCAAGCCCC
AACTTTCCCG  ACCACGGTCG  GTGTCTGCTG  GGTACCCCGC  AATTCCCCAG
TCACTCCCGC  CCAAATCACC  AAGACCTATG  GCCCACTTGA  AATGATGAAC
CCCCGGGTCA  AAGATTGAT  CTCACCCCTC  ATCGTCAAGT  GCCCACTTGA
AATGATGAAC  CCCCCGGTCA  AAGATTGAT  TCAGTACCTT  GACTCGCCCA
AACTCCTCAT  CTCCATCACC  GCTCAACCCA  CCGCTCCCCC  CCGATCGACC
TGCATAATAA  CTGTATCAGG  AACTCTCTCG  ATGCACTCTC  CGCTCATCAC
GGACACTTCC  ACCTAAGTTC  TCGATCTTTA  AAATC

                                L:T
TTTTTGTTTG  ATGGAGAATT  GCGCAGAGGG  GTTATATCTG  CGTGAGGATC
TGTCACTCCG  CCGTGTGGGA  TACCTCCCTG  CTAAGGCCCC  TTGAGTGATG
TTCCCTCGGA  CTGGGGACCG  CTGGCTTCCG  AGCTATGTCC  CCGATACCAT
GGAATTTCGA  CTCATTTGAG  CCCCCGCTCA  GFTTGCTAGC  AGAACCCGGC
ACATGGTTCG  CCGATACCAT  GGAGTTTCGA  AAGAAACACT  CTGTTAGGTG
GTATGAGTCA  TGACCGACCC  ACCGAGACCC  TAAGGCTTAT  CCTATGCTGA
TCTCCGTGAA  TGTCTATCAT  TCCTACACAG  GACCC

                                L:SK
TTATCAGGAA  CTCTCTCGAT  GCACTCTCCG  CTCATCACGG  ACACTTCCAC
CTAAGTTCTC  GATCTTTAAA  ATCGTTAGCT  CGCCAGTTAG  CGAGGICTGT
CCCCACAGGA  CAGATAATCG  GGTGCAACTC  CCGCCCTCT  TCCGAGGGTC
ATCGGAACC
```

4

1

가

0.3-0.5mm

5%

가 가

flask

가

가 가

RT-PCR

가

가

ELISA





	3-5	ELISA	magnetic bead	12
30-1	plate	ELISA conjugate	coating	가
	가	10	가	2
	가	NC filter conjugate	blotting system	가
	1	가		
	biotin-avidin	23%	가	

< 9 >

Method	Theory	Sensitivity	Time(hr)
I. ELISA	direct indirect	100	3-5
II. magnetic bead	mAb coating on surface	118	overnight
III. biotin-avidin	indirect ELISA mAb-biotin avidin-enzyme bridged avidin- biotin	115	5
IV. II+III		123	overnight
V. Nitrocellulose filter paper	II+III	120	5

\* filter paper  
paper

가

2.

가

tracer conjugate  
polystyrene tube ELISA reader 가  
가 가

< 10> Kit

	content		vol. / bottle
I. mAb	to LSV	5	1ng/ vial
	to TBV	2	
	to LSV, TBV	1	
II. Conjugate	mAb 3	alkaline phosphatase biotin	10ug/ mAb/ vial
III. diluent / blocking sol.		TBS buffer	50ml
		gelatin	20ml
		10%BSA	
IV. 10X buffer		TBS - 0.05% Tween-20	100ml
V. polystyrene tube			
VI. Protocol			

Kit

가 Polystyrene tube 96 well plate . 96well plate 가 .

3. ELISA

= =

가

sample buffer homogenate

20000rpm

40000rpm 3

< 11 > 4

10g

ELISA

	A	B	C	D	E
SV-4	0.015	0.81	0.05	0.62	0.01
SV-7	-	0.23	-	0.31	-
SV-11	0.11	0.18	-	0.22	0.39
SV-15	0.50	0.45	-	0.01	-
SV-20	0.82		-	0.23	-

Max. OD = 1.0

가

가

가

2

가

< 12 >

		NO of detection					(%)
		Antibodies to virus					
		sv-4	sv-7	sv-11	sv-15	sv-20	
( )	52	12			21	2	67.3
A	11		7				63.6
B	20			8			40
C	10				7		70
D	30		5	6		10	70

가

Sample NO	Isolates	Symptom	Virus	Antibodies to virus				
				sv-4	sv-7	sv-11	sv-15	sv-20
SV-1	SJ-10-2				++			
SV-2	SJ-10-3		-					
SV-3	SJ-10-8	,		+++				
SV-4	SJ-1022			+++		++		
SV-5	SJ-16-5							
SV-6	SJ-16-7			++				
SV-7	SC-3-11	,			+++			
SV-8	SC-5-8		-					
SV-9	SC-5-16		-					
SV-10	SC-7-3					++		+
SV-11	SC-9-11	,				+++		++
SV-12	SC-10-5				++			
SV-13	SJ-12-3	chlorosis	-					
SV-14	SJ-12-7	wild twist				+		
SV-15	SJ-12-6	curling					+++	

: +, ++, +++.

Sample NO	Isolates	Symptom	Virus	Antibodies to virus				
				sv-4	sv-7	sv-11	sv-15	sv-20
SV-16	SJ-13-2			++				
SV-17	SB-1-3		-				+	+++
SV-18	SB-2-6	curling	-	+++				
SV-19	SK-1-4		-			+		++
SV-20	SK-1-7					++		+++
SV-21	SJ-13-2	,	,				++	
SV-22	SJ-12-4		-	+			+	
SV-23	SJ-11-8	chlorosis	-					
SV-24	SJ-8-12	wild twist				++		+
SV-25	SJ-12-5	curling				++		
SV-26	SJ-19-7				+++			
SV-27	SC-13-11		-				+	++
SV-28	SC-15-8	curling						+
SV-29	SC-15-16						++	
SV-30	SC-17-3				++		+	+

: +, ++, +++.

4.

. 1

가



< 27 >



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