

# Evaluating Variability of Allergens in Commodity Crops (Peanuts)

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# The Primary Allergens of Peanut Are the Seed Storage Proteins

- Ara h 1 - 7S vicilin-type globulin (conarachin)
- Ara h 2 - conglutin-type 2S albumin  
Other conglutins - Ara h 6, Ara h 7
- Ara h 3/4 - 11S legumin-type globulin (arachin)

# Other Allergenic Seed Proteins

Ara h 5 - profilin

Ara h 8 - pathogenesis-related (PR-10)

Oleosin

Lipid transfer protein

Agglutinin

Eight Allergenic Proteins?

How many genes for each protein class?

How many alleles for each gene?

# Peanut Seed Storage Proteins

peanut seeds are ~25% protein

## Ara h 1 and Ara h 2

Both are recognized by serum IgE from >90% patients with peanut allergies.

**Ara h 1** - 7-18.5% of total protein (Kang et al. 2007. Crop Sci. 47:997)

**Ara h 2** - 6.2-13.2%

## Ara h 3

Recognized by ~50% of patient sera

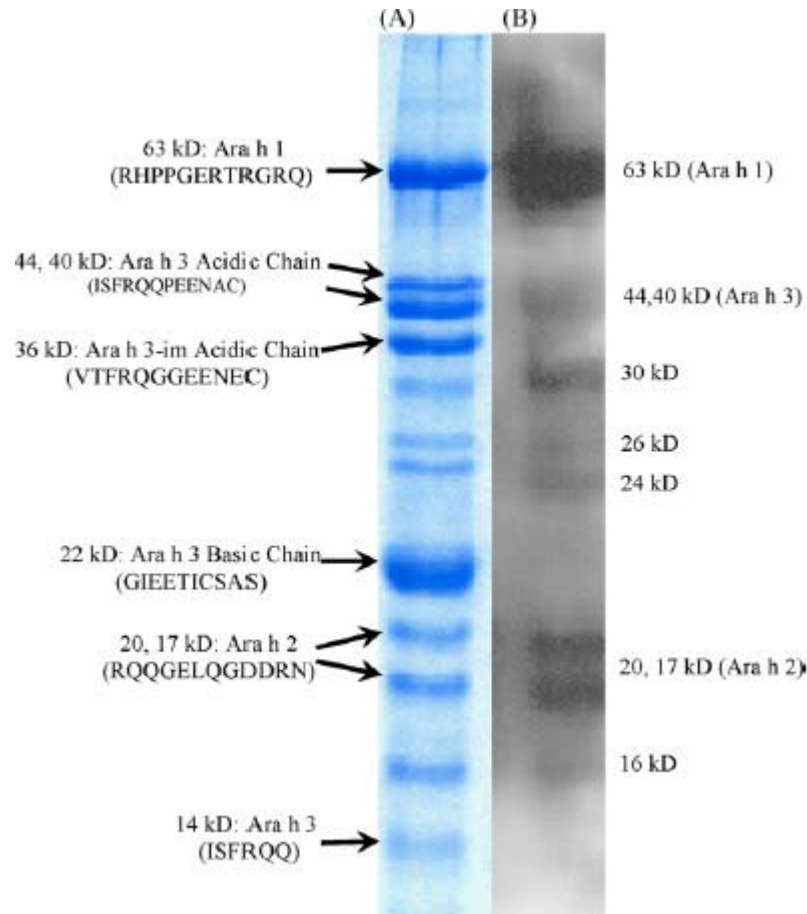
21.8-38.5% of total protein

## Ara h 6

Recognized by ~70% of patient sera (Koppelman et al. 2005. Clin. Exper. Allergy 35:490)

# Glycinin Proteins in Peanut

Kang and Gallo. 2007. Plant Sci. 172:345



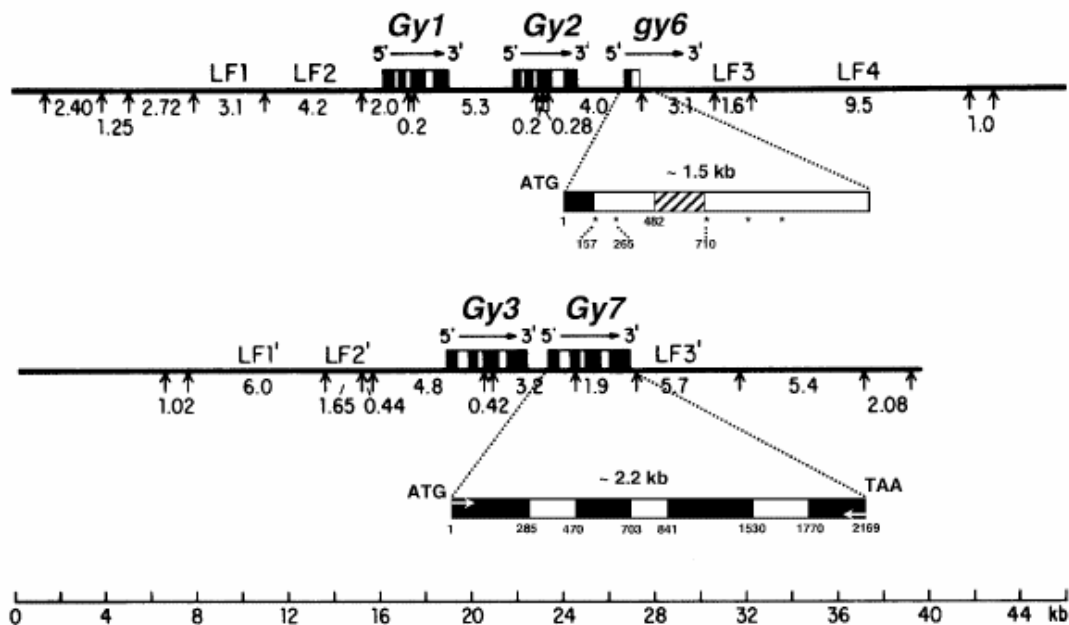
# Glycinin Genes in Soybean

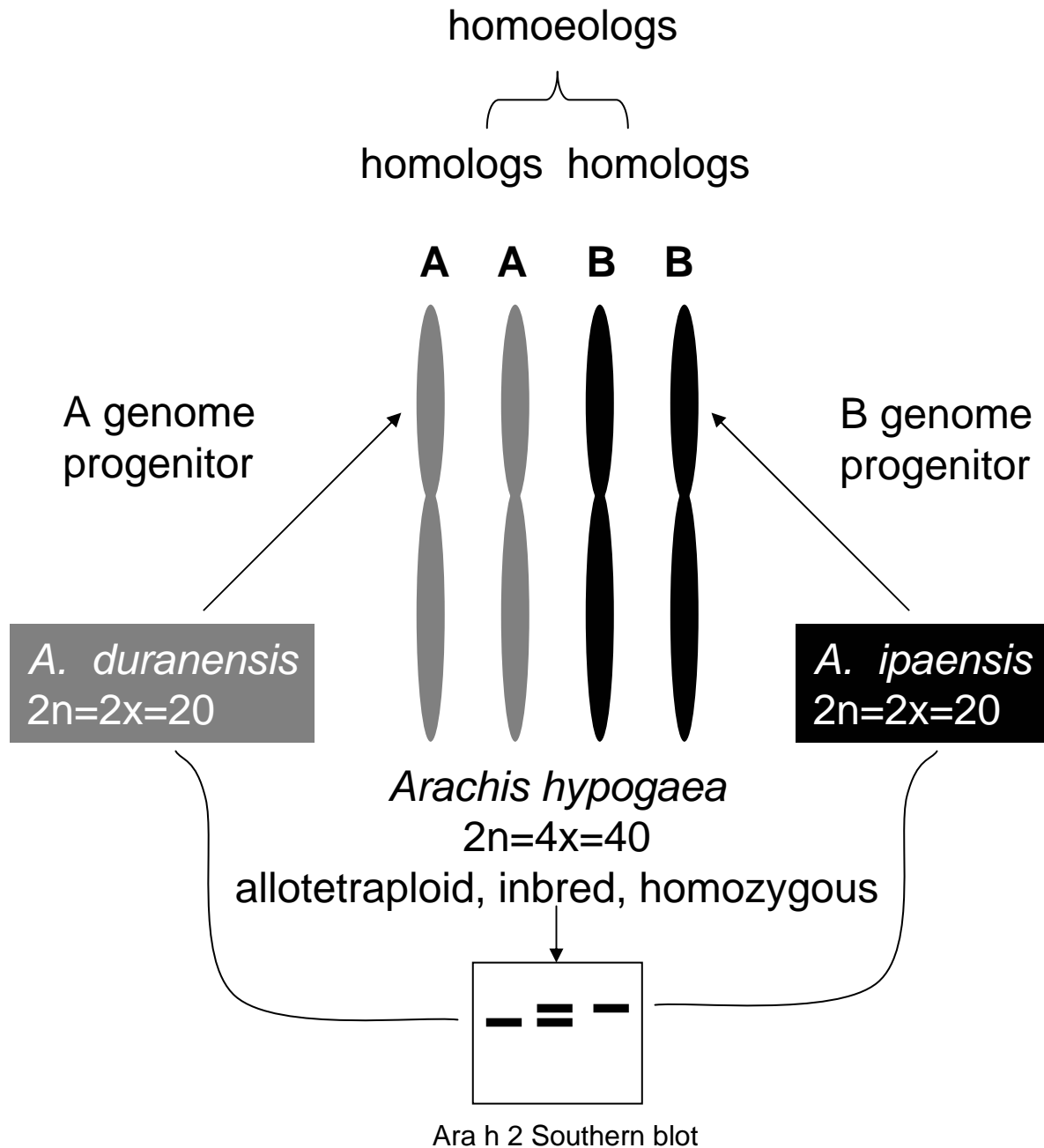
Beilinson et al. 2002. TAG 104:1132

Class 1 - *Gy* 1, 2, 3 - linkage groups N & L

Class 2 - *Gy* 4, 5 - linkage groups O & F

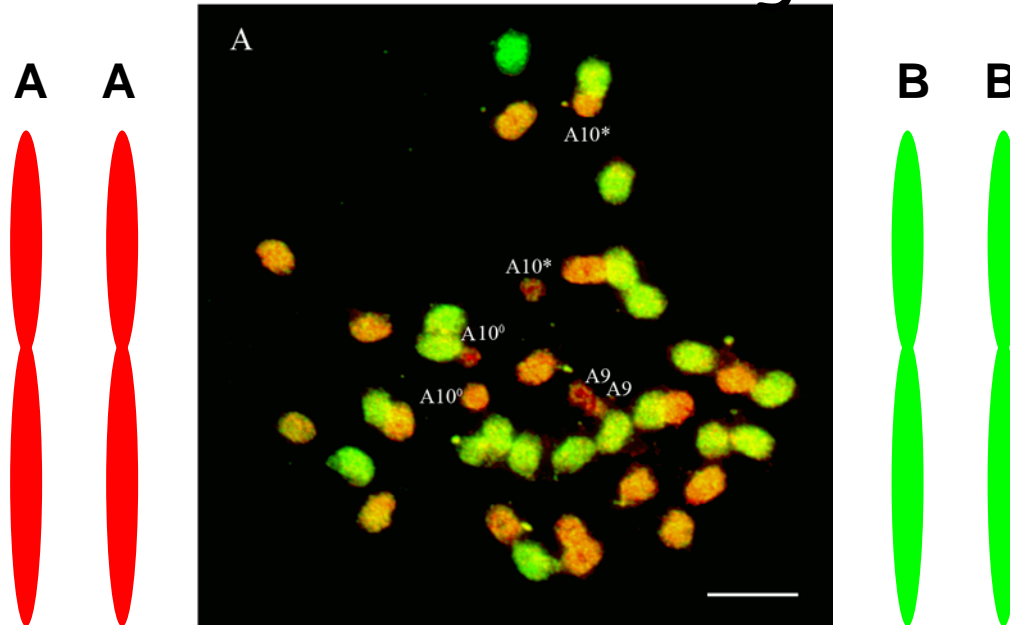
*gy*6, *Gy* 7







# Peanut is an allotetraploid species with two subgenomes



- A subgenome (*A. duranensis*)
- B subgenome (*A. ipaensis*)

Ramos *et al.*, 2006. *Mol Gen Genomics*. 275: 578-592.

L77197 (Stanley, J.S.) (97) CCAGCTCGAGAGGGCGAACCTGAGGCCCTGCGAGCAACATCTCATGCAGAAAG  
 AY722889 (CON G2-Arah2) (105) CCAGCTCGAGAGGGGCGAACCTGAGGCCCTGCGAGCAACATCTCATGCAGAAAG  
 Ara h2 cDNA (A-genome) (105) CCAGCTCGAGAGGGGCGAACCTGAGGCCCTGCGAGCAACATCTCATGCAGAAAG  
 AY158467 (Chatel et al.) (105) CCAGCTCGAGAGGGGCGAACCTGAGGCCCTGCGAGCAACATCTCATGCAGAAAG  
 Ara h2 cDNA (B-genome) (105) CCAGCTCGAGAGGGGCGAACCTGAGGCCCTGCGAGCAACATCTCATGCAGAAAG  
 AY581853(CON G1-Arah2.02) (105) CCAGCTCGAGAGGGGCGAACCTGAGGCCCTGCGAGCAACATCTCATGCAGAAAG  
 AY117434(Becker et al.) (96) CCAGCTCGAGAGGGGCGAACCTGAGGCCCTGCGAGCAACATCTCATGCAGAAAG  
 Consensus (105) CCAGCTCGAGAGGGGCGAACCTGAGGCCCTGCGAGCAACATCTCATGCAGAAAG

Section 4

L77197 (Stanley, J.S.) (149) ATCCAACGTGACGAGGATTTCATATGACCGGGACCCGTACAGCCCTAGTTCAGG  
 AY722889 (CON G2-Arah2) (157) ATCCAACGTGACGAGGATTTCATATGACCGGGACCCGTACAGCCCTAGTTCAGG  
 Ara h2 cDNA (A-genome) (157) ATCCAACGTGACGAGGATTTCATATGACCGGGACCCGTACAGCCCTAGTTCAGG  
 AY158467 (Chatel et al.) (157) ATCCAACGTGACGAGGATTTCATATGACCGGGACCCGTACAGCCCTAGTTCAGG  
 Ara h2 cDNA (B-genome) (157) ATCCAACGTGACGAGGATTTCATATGACCGGGACCCGTACAGCCCTAGTTCAGG  
 AY581853(CON G1-Arah2.02) (157) ATCCAACGTGACGAGGATTTCATATGACCGGGACCCGTACAGCCCTAGTTCAGG  
 AY117434(Becker et al.) (148) ATCCAACGTGACGAGGATTTCATATGACCGGGACCCGTACAGCCCTAGTTCAGG  
 Consensus (157) ATCCAACGTGACGAGGATTTCATATGACCGGGACCCGTACAGCCCTAGTTCAGG

Section 5

L77197 (Stanley, J.S.) (201) ATCC-----GTACAGCCCTAG  
 AY722889 (CON G2-Arah2) (209) ATCC-----GTACAGCCCTAG  
 Ara h2 cDNA (A-genome) (209) ATCC-----GTACAGCCCTAG  
 AY158467 (Chatel et al.) (209) ATCCGTACAGCCCTAGTCAGGACCCGGACAGACGTGATCCGTACAGCCCTAG  
 Ara h2 cDNA (B-genome) (209) ATCCGTACAGCCCTAGTCAGGACCCGGACAGACGTGATCCGTACAGCCCTAG  
 AY581853(CON G1-Arah2.02) (209) ATCCGTACAGCCCTAGTCAGGACCCGGACAGACGTGATCCGTACAGCCCTAG  
 AY117434(Becker et al.) (200) ATCCGTACAGCCCTAGTCAGGACCCGGACAGACGTGATCCGTACAGCCCTAG  
 Consensus (209) ATCCGTACAGCCCTAGTCAGGACCCGGACAGACGTGATCCGTACAGCCCTAG

Section 6

L77197 (Stanley, J.S.) (217) TCCATATGATCGGAGAGGCGCTGGATCCTCTCAGCACCAAGAGAGGTTGTTGC  
 AY722889 (CON G2-Arah2) (225) TCCATATGATCGGAGAGGCGCTGGATCCTCTCAGCACCAAGAGAGGTTGTTGC  
 Ara h2 cDNA (A-genome) (225) TCCATATGATCGGAGAGGCGCTGGATCCTCTCAGCACCAAGAGAGGTTGTTGC  
 AY158467 (Chatel et al.) (261) TCCATATGATCGGAGAGGCGCTGGATCCTCTCAGCACCAAGAGAGGTTGTTGC  
 Ara h2 cDNA (B-genome) (261) TCCATATGATCGGAGAGGCGCTGGATCCTCTCAGCACCAAGAGAGGTTGTTGC  
 AY581853(CON G1-Arah2.02) (201) TCCATATGATCGGAGAGGCGCTGGATCCTCTCAGCACCAAGAGAGGTTGTTGC  
 AY117434(Becker et al.) (202) TCCATATGATCGGAGAGGCGCTGGATCCTCTCAGCACCAAGAGAGGTTGTTGC  
 Consensus (261) TCCATATGATCGGAGAGGCGCTGGATCCTCTCAGCACCAAGAGAGGTTGTTGC

Section 7

# Partial Amino Acid Sequence Alignment of A- and B-genome ara h 2 Genes

		Section 1				
		(1)	1	10	20	30 49
Tsl L77197 (Stanley, J.S.)	(1)	---	LTILVAL	LALFL	LAHA	SARQQWELQGDRRCQS
Tsl cDNA Ara h2 (A-genome)	(1)	MAK	LTILVAL	LALFL	LAHA	SARQQWELQGDRRCQS
Tsl cDNA Ara h2 (B-genome)	(1)	MAK	LTILVAL	LALFL	LAHA	SARQQWELQGDRRCQS
Tsl cDNA AY117434 (Becker et al)	(1)	---	LTILVAP	LALFL	LAHA	SARQQWELQGDRRCQS
Tsl cDNA AY158467 (Chatel et al)	(1)	MAK	LTILVAL	LALFL	LAHA	SARQQWELQGDRRCQS
Consensus	(1)	MAK	LTILVAL	LALFL	LAHA	SARQQWELQGDRRCQS
		Section 2				
		(50)	50	60	70	80 98
Tsl L77197 (Stanley, J.S.)	(47)	MQKIQRDED	SYER	DPYSP	SQDPYSP	-----SPYDRRGAGSSQ
Tsl cDNA Ara h2 (A-genome)	(50)	MQKIQRDED	SYER	DPYSP	SQDPYSP	-----SPYDRRGAGSSQ
Tsl cDNA Ara h2 (B-genome)	(50)	MQKIQRDED	SYGR	DPYSP	SQDPYSP	SQDPDRRDPYSPSPYDRRGAGSSQ
Tsl cDNA AY117434 (Becker et al)	(47)	MQKIQRDED	SYGR	DPYSP	SQDPYSP	SQDPDRRDPYSPSPYDRRGAGSSQ
Tsl cDNA AY158467 (Chatel et al)	(50)	MQKIQRDED	SYGR	DPYSP	SQDPYSP	SQDPDRRDPYSPSPYDRRGAGSSQ
Consensus	(50)	MQKIQRDED	SYGR	DPYSP	SQDPYSP	SQDPDRRDPYSPSPYDRRGAGSSQ
		Section 3				
		(99)	99	110	120	130 147
Tsl L77197 (Stanley, J.S.)	(84)	HQERCCNEL	NEFENN	QRCMCEAL	QQIMENQSDRL	QGRQQEQQFKREL
Tsl cDNA Ara h2 (A-genome)	(87)	HQERCCNEL	NEFENN	QRCMCEAL	QQIMENQSDRL	QGRQQEQQFKREL
Tsl cDNA Ara h2 (B-genome)	(99)	HQERCCNEL	NEFENN	QRCMCEAL	QQIMENQSDRL	QGRQQEQQFKREL
Tsl cDNA AY117434 (Becker et al)	(96)	HQERCCNEL	NEFENN	QRCMCEAL	QQIMENQSDRL	QGRQQEQQFKREL
Tsl cDNA AY158467 (Chatel et al)	(99)	HQERCCNEL	NEFENN	QRCMCEAL	QQIMENQSDRL	QGRQQEQQFKREL
Consensus	(99)	HQERCCNEL	NEFENN	QRCMCEAL	QQIMENQSDRL	QGRQQEQQFKREL
		Section 4				
		(148)	148	160 *	173	
Tsl L77197 (Stanley, J.S.)	(133)	LPQQCGLRAP	QRCDL	D	VESGG	RDY-
Tsl cDNA Ara h2 (A-genome)	(136)	LPQQCGLRAP	QRCDL	D	VESGG	RDY-
Tsl cDNA Ara h2 (B-genome)	(148)	LPQQCGLRAP	QRCDL	E	VESGG	RDY-
Tsl cDNA AY117434 (Becker et al)	(145)	LPQQCGLRAP	QRCDL	E	VESGG	RDY-
Tsl cDNA AY158467 (Chatel et al)	(148)	LPQQCGLRAP	QRCDL	E	VESGG	RDY-
Consensus	(148)	LPQQCGLRAP	QRCDL	E	VESGG	RDY-

- There are 2 copies of *ara h 2* in tetraploid peanut
- *ara h 2* is similar to another allergen gene, *ara h 6*

arah2-arah6 (8A2a).apr

Section 1

	(1)	1	10	20	30	40	50	60	70	88	
Arah6 ORF (8A2a)	(1)	ATGGCCAAGTC	CACCATC	CTGGTAGCT	CTCCTTGCCCT	TCGTCCTG	GTG	GCACACGC	CTCCGC	AATGAGGC	GCGAGAGGGGGAGACAGG
Arah2 ORF (8A2a)	(1)	ATGGCCAAGCT	CACCAT	CTAGTAGC	CTCGCCCTTT	TCCTCCTG	GCT	GCCCACGC	ATCTGC	---	GAGGCAGCAGTGGGAAC
Consensus	(1)	ATGGCCAAG	CACCAT	CTGTAGC	CTC	TCCTG		GCACGC	TCGC	GAGGC	AGGGACA

Section 2

	(89)	89	100	110	120	130	140	150	160	176	
Arah6 ORF (8A2a)	(89)	GGGACTCATC	AAGCTGC	GAGAGG	CAGGTAGAC	CAGGGTT	AACCTCA	AGCCCTG	CGAGCAGCA	CAATATGC	CAGAGGATC
Arah2 ORF (8A2a)	(86)	GAGACA---	GAAAGATGC	CAGAGC	CAGCTCGAG	AGGGCG	AACCTTAG	GCCCTG	CGAGCAACA	TCATGC	CAGAAAATC
Consensus	(89)	G GAC	AAG TGC	AGAG CAG	T GA AGGG	AACCT A	GCCCTG	CGAGCA CA	T ATGCAGA	ATC	

Section 3

	(177)	177	190	200	210	220	230	240	250	264
Arah6 ORF (8A2a)	(166)	---	ATGGGCGAG	---	---	CAAGAG	CAGTAC	---	GAC	---
Arah2 ORF (8A2a)	(171)	GGATTTCAT	ATGGACGGG	ACCCGTACAG	CCCTAGT	CAGGATC	CGTAC	AGCCCTAGT	CAGGAC	CCGGACAGACGTGA
Consensus	(177)		ATGG CG G			CA GA C	GTAC		GAC	TCC

Section 4

	(265)	265	270	280	290	300	310	320	330	340	352
Arah6 ORF (8A2a)	(193)	---	TACGAT	ATTAGGAGT	ACTCGATC	CTCG	ACCAGCAAC	CAGAGGTG	TGCGATGAG	CTGAACGAGAT	TGGAGAACA
Arah2 ORF (8A2a)	(259)	AGTCCA	TATGAT	CGGAGAGG	CGCTG	GATCCTC	TCAGCAC	CAAGAGAGGTG	TGCAATGAG	CTGAACGAGTT	TGAGAACA
Consensus	(265)		TA GAT	AG G	CT GATCCTC		A CA CAA	AGAGGTG	TGC ATGAG	CTGAACGAG	T GAGAACA

Section 5

	(353)	353	360	370	380	390	400	410	420	430	440
Arah6 ORF (8A2a)	(275)	GCATGTGCGAGG	CATTGCA	GCAGATAA	TGGAGAAC	CCAGT	GCGATAGG	TTCAGG	ACAGGCAA	ATGGTGCA	GCAGTTC
Arah2 ORF (8A2a)	(347)	GCATGTGCGAGG	CATTGCA	ACAGATAA	TGGAGAAC	CCAGAG	GCGATAGG	TTCAGG	GGAGGCAA	CAAGGAGCA	CAGTTC
Consensus	(353)	GCATGTGCGAGG	CATTGCA	CAGATAA	TGGAGAAC	CCAG	GCGATAGG	TTCAGG	AGGCAA	GG GCA	CAGTTC

Section 6

	(441)	441	450	460	470	480	490	500	510	522
Arah6 ORF (8A2a)	(363)	CATGA	ACTTGCC	CAACAGT	GTAACT	TTAGGG	CACCACAG	CGTTGCGA	TTTGGA	CGTG---
Arah2 ORF (8A2a)	(435)	CAGGA	ACTTGCC	CAACAGT	GTGCGG	CTCAGGG	CACCACAG	CGTTGCGA	CTTGGA	AGTCGAA
Consensus	(441)	CA GA	ACTTGCC	CAACAGT	G	C T	AGGGC	ACCACAG	CGTTGCGA	TTGGA GT

- There are 3 copies of *ara h 6* in tetraploid peanut

# Arah6 genes.apr

Section 1

119

Section 2

238

Section 3

357

Section 4

476

Section 5

595

Section 6

639

Section 7

639

Section 8

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Section 9

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Section 10

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Section 13

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Section 14

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Section 15

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Section 16

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Section 17

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Section 18

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Section 19

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Section 20

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# Three Amino Acid Changes Distinguish A- and B-Genome *ara h 6* Genes

	(1)	1	10	20	30	45
PSC33 conglutin	(1)	-AKSTILVALLALVLVAHASAMRRERGRQGDSSSCERQVDRVNLK				
Translation of AY848699 (CONG4-Psc33)	(1)	MAKSTILVALLALVLVAHASAMRRERGRQGDSSSCERQVDRVNLK				
Translation of AD 6,1arah6	(1)	MAKSTILVALLALVLVAHASAMRRERGRQGDSSSCERQVDRVNLK				
Translation of 4,10 arah6	(1)	MAKSTILVALLALVLVAHASAMRRERGRQGDSSSCERQVDRVNLK				
Translation of AY849314 (CONG5-Arah6)	(1)	MAKSTILVALLALVLVAHASAMRRERGRQGDSSSCERQVDRVNLK				
Translation of AY722690 (CONG3)	(1)	MAKSTILVALLALVLVAHASAMRRERGRQGDSSSCERQVDRVNLK				
protein arah6	(1)	-----AHASAMRRERGRQGDSSSCERQVDRVNLK				
Translation of AY871100	(1)	-----MRRERGRQGDSSSCERQVDRVNLK				
Translation of AI 5,1arah6	(1)	MAKSTILVALLALVLVAHASAMRRERGRQGDSSSCERQVDRVNLK				
Translation of 4,6 arah6	(1)	MAKSTILVALLALVLVAHASAMRRERGRQGDSSSCERQVDRVNLK				
Consensus	(1)	MAKSTILVALLALVLVAHASAMRRERGRQGDSSSCERQVDRVNLK				
Section 2						
	(46)	46	60	70	80	90
PSC33 conglutin	(45)	PCEQHIMQRIMGEQEYDSYDIRSTRSSDQQQRCCDELDQMENTE				
Translation of AY848699 (CONG4-Psc33)	(46)	PCEQHIMQRIMGEQEYDSYDIRSTRSSDQQQRCCDELDQMENTE				
Translation of AD 6,1arah6	(46)	PCEQHIMQRIMGEQEYDSYDIRSTRSSDQQQRCCDELDQMENTE				
Translation of 4,10 arah6	(46)	PCEQHIMQRIMGEQEYDSYDIRSTRSSDQQQRCCDELDQMENTE				
Translation of AY849314 (CONG5-Arah6)	(46)	PCEQHIMQRIMGEQEYDSYDIRSTRSSDQQQRCCDELNEMENTQ				
Translation of AY722690 (CONG3)	(46)	PCEQHIMQRIMGEQEYDSYDIRSTRSSDQQQRCCDELNEMENTQ				
protein arah6	(30)	PCEQHIMQRIMGEQEYDSYDFGSTRSSDQQQRCCDELNEMENTQ				
Translation of AY871100	(25)	PCEQHIMQRIMGEQEYDSYDIRSTRSSDQQQRCCDELNEMENTQ				
Translation of AI 5,1arah6	(46)	PCEQHIMQRIMGEQEYDSYDIRSTRSSDQQQRCCDELNEMENTQ				
Translation of 4,6 arah6	(46)	PCEQHIMQRIMGEQEYDSYDIRSTRSSDQQQRCCDELNEMENTQ				
Consensus	(46)	PCEQHIMQRIMGEQEYDSYDIRSTRSSDQQQRCCDELNEMENTQ				
Section 3						
	(91)	91	100	110	120	135
PSC33 conglutin	(90)	RCMCEALQQIMENQCDRLQDRQM VQ QFKRELMNLPQQCNFRAPQR				
Translation of AY848699 (CONG4-Psc33)	(91)	RCMCEALQQIMENQCDRLQDRQM VQ QFKRELMNLPQQCNFRAPQR				
Translation of AD 6,1arah6	(91)	RCMCEALQQIMENQCDRLQDRQM VQ QFKRELMNLPQQCNFRAPQR				
Translation of 4,10 arah6	(91)	RCMCEALQQIMENQCDRLQDRQM VQ QFKRELMNLPQQCNFRAPQR				
Translation of AY849314 (CONG5-Arah6)	(91)	RCMCEALQQIMENQCDRLQDRQM VQ QFKRELMNLPQQCNFRAPQR				
Translation of AY722690 (CONG3)	(91)	RCMCEALQQIMENQCDRLQDRQM VQ QFKRELMNLPQQCNFRAPQR				
protein arah6	(75)	RCMCEALQQIMENQCDGLQDRQM VQ QFKRELMNLPQQCNFGAPQR				
Translation of AY871100	(70)	RCMCEALQQIMENQCDRLQDRQM VQ QFKRELMNLPQQCNFRAPQR				
Translation of AI 5,1arah6	(91)	RCMCEALQQIMENQCDRLQDRQM VQ QFKRELMNLPQQCNFRAPQR				
Translation of 4,6 arah6	(91)	RCMCEALQQIMENQCDRLQDRQM VQ QFKRELMNLPQQCNFRAPQR				
Consensus	(91)	RCMCEALQQIMENQCDRLQDRQM VQ QFKRELMNLPQQCNFRAPQR				

# Targeting Induced Local Lesions IN Genomes

(McCallum et al. 2000. Plant Physiol. 123:439)

## Functional Genomics Strategy

Forward genetics: Trait  $\rightarrow$  Gene  
Ex: mutant phenotype

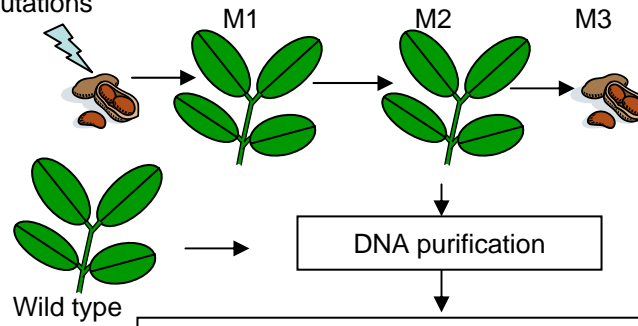
Reverse genetics: Gene  $\rightarrow$  Trait  
Ex: RNAi; gene knockout

# Advantages of TILLING

- Yields point mutations
- Mutation can be in any gene (random)
- Can target screening to gene of interest
- Phenotype can be determined later



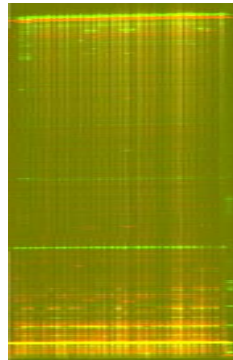
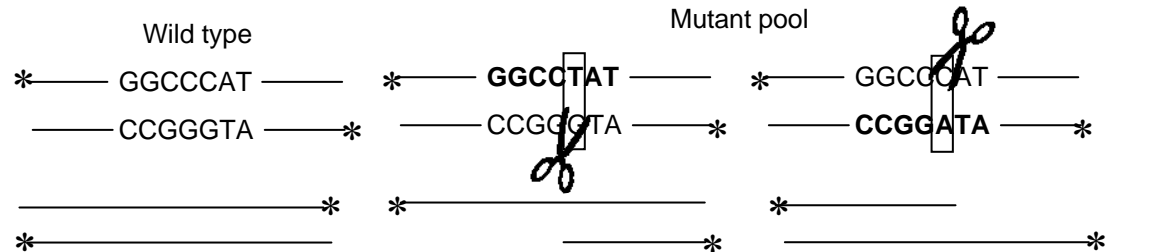
EMS induces G/C-to-A/T  
single nucleotide mutations



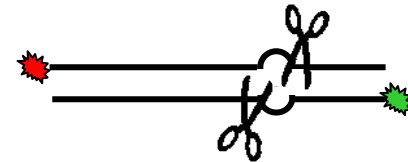
DNA purification

PCR with 5'-end-labeled gene-specific primers \*

Heating and cooling DNA to form heteroduplexes between wild type  
and mutant amplicons

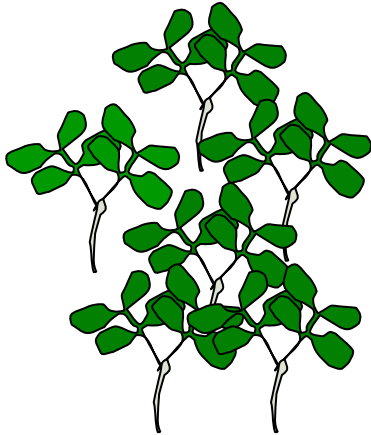


Denaturing polyacrylamide gels



# Screening Is Carried Out By Mismatch Detection With Cel I

Screen pools of DNA  
from multiple plants  
by PCR



atg<sup>g</sup>cttgt  
tac<sup>c</sup>gaaca

atg<sup>a</sup>cttgt  
tac<sup>t</sup>gaaca

tac<sup>c</sup>gaaca  
atg<sup>a</sup>cttgt

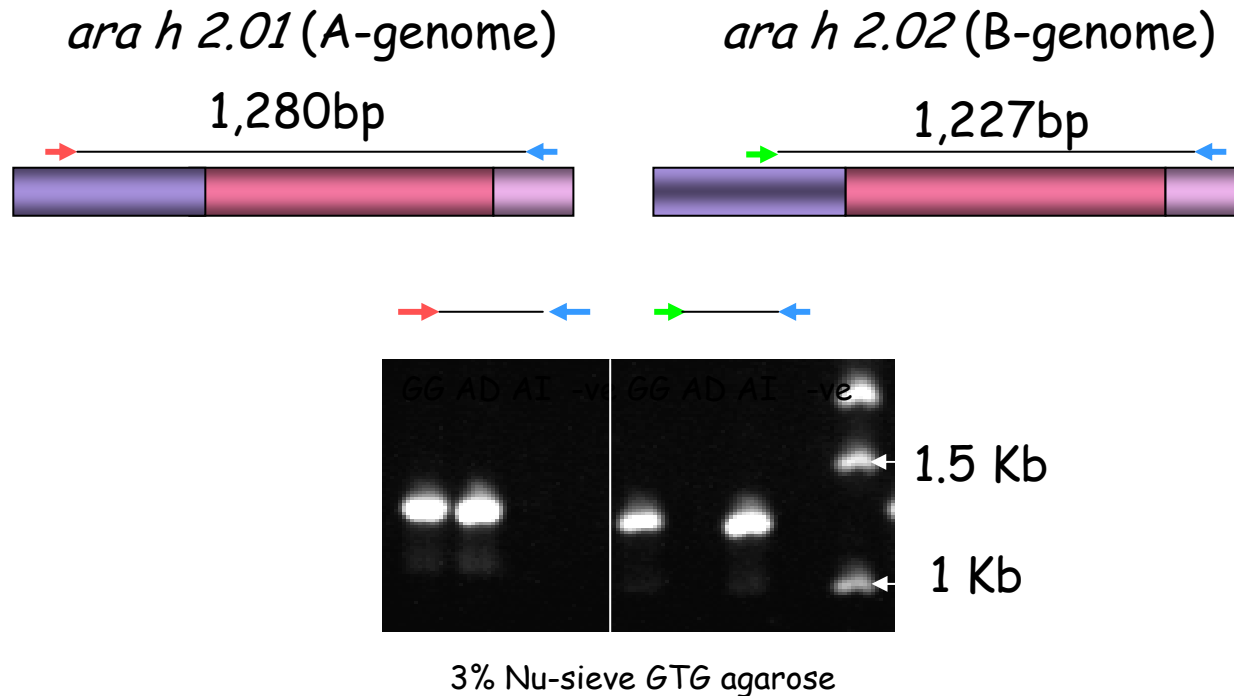
atg<sup>g</sup>cttgt atg<sup>g</sup>cttgt  
tac<sup>c</sup>gaaca tac<sup>c</sup>gaaca

atg<sup>a</sup>cttgt  
tac<sup>t</sup>gaaca tac<sup>t</sup>gaaca  
atg<sup>a</sup>cttgt

tac<sup>t</sup>gaaca  
atg<sup>g</sup>cttgt

tac<sup>c</sup>gaaca  
atg<sup>g</sup>cttgt

# Gene-specific PCR for A- and B-Genome Ara h 2 Genes



Ozias-Akins *et al.*, 2006. Hypoallergenic foods beyond infant formulas. In: Food Allergy. ASM Press, Herndon, VA.

TILLING



Chemically-mutagenized  
populations

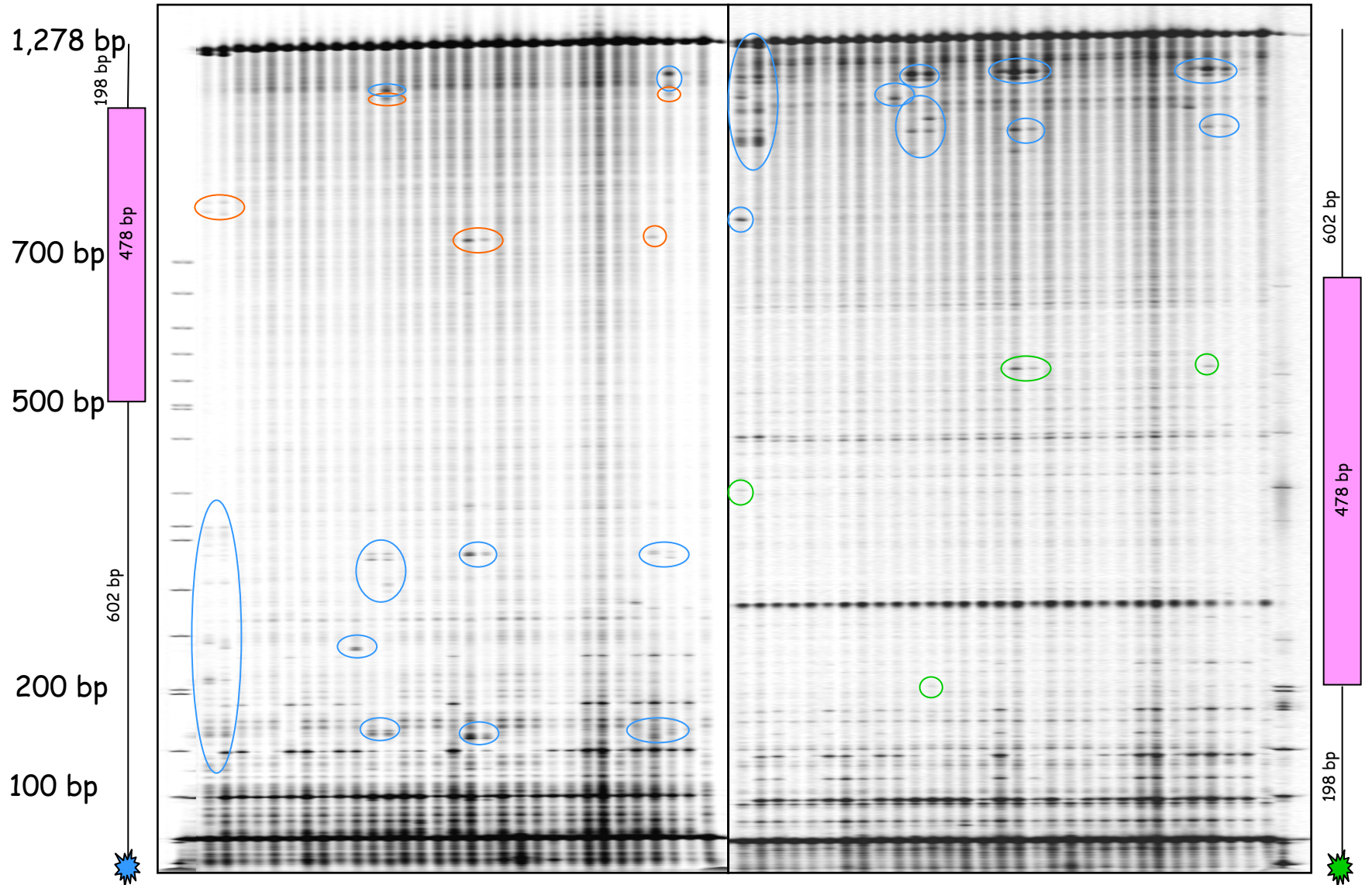
ECOTILLING



Natural populations

# Ecotilling

## *Arachis duranensis* accessions

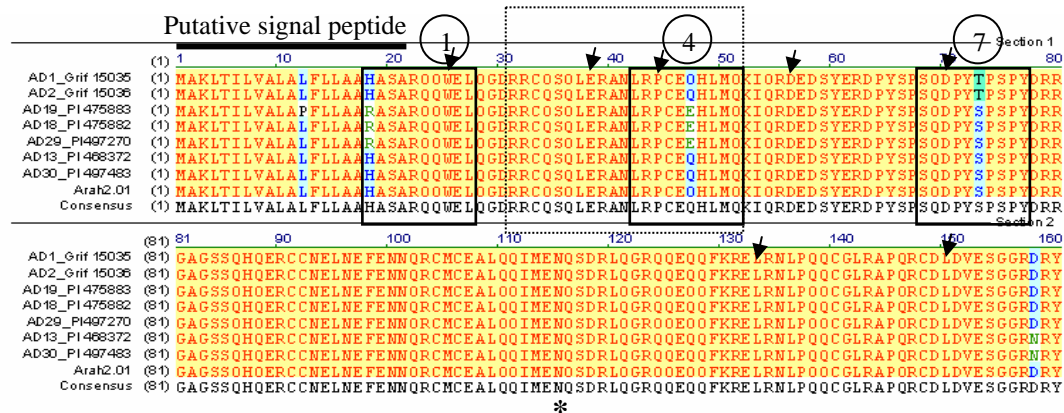


○ Putative mutations in ORF    ○ Putative mutations in 5'UTR    ○ Putative mutations in 3'UTR

# Ecotilling of *Arachis duranensis*

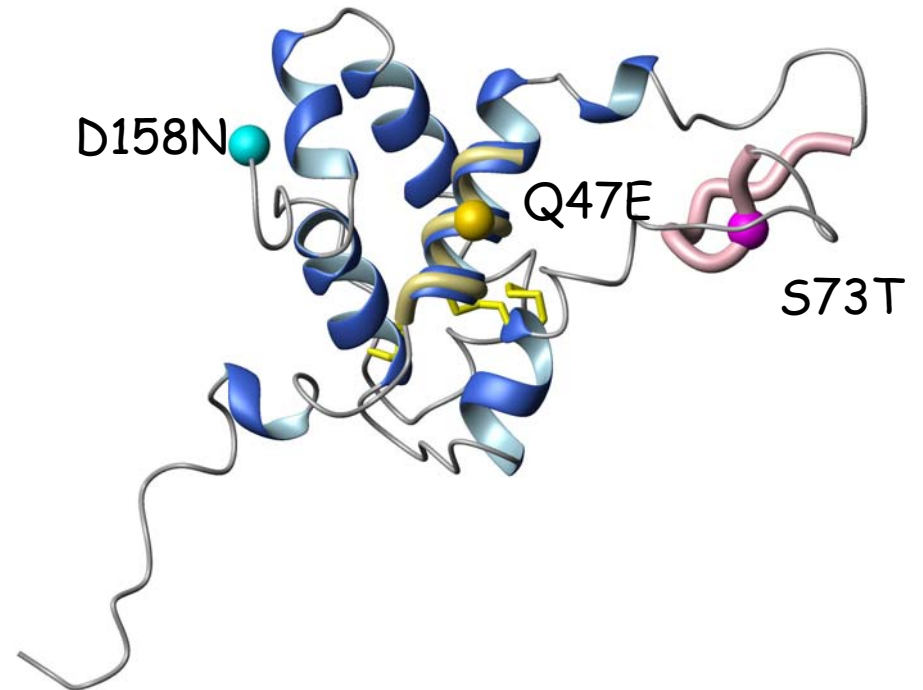
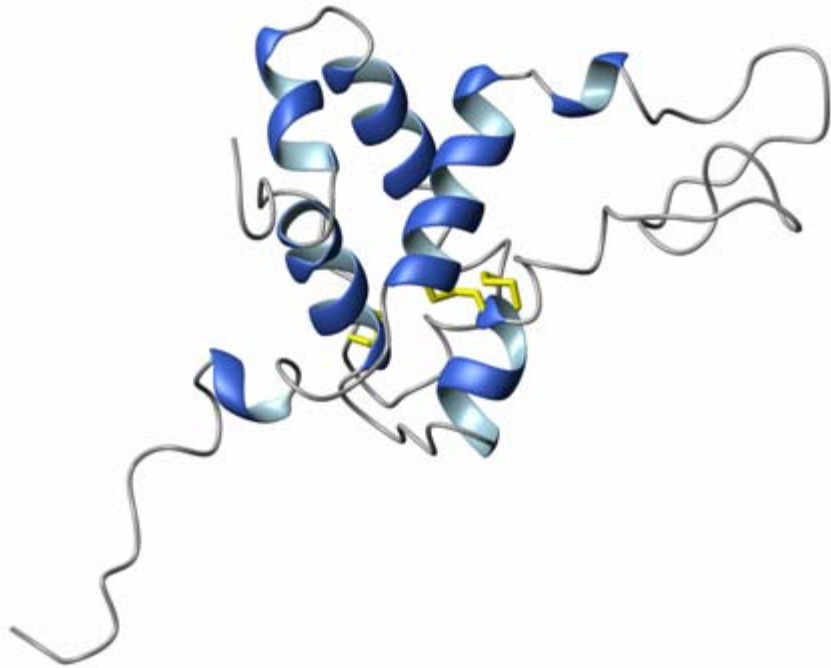
- Screened 30 accessions
- Seven showed variation from the WT coding region
- Eight SNPs total - 3 silent, 5 missense
- Indels in non-coding regions

# Amino Acid Changes Detected in *ara d 2.01*



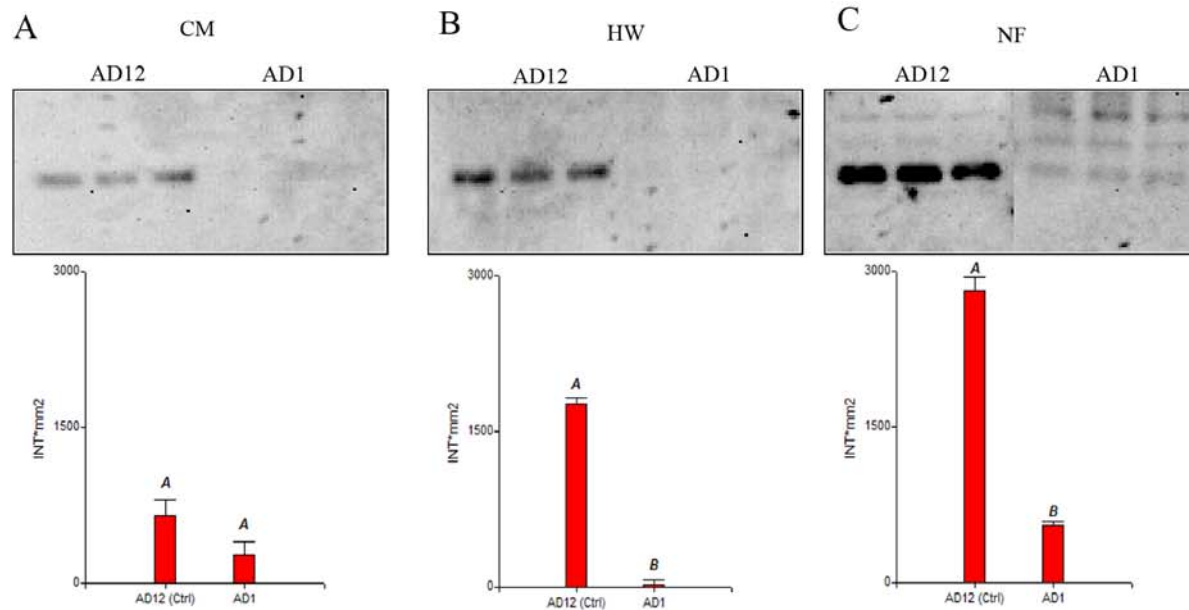
SNP position	aa change	Nature of the mutation	IgE epitope affected	T-cell epitope affected <sup>#</sup>	SIFT score	<i>A. duranensis</i> accession	Short ID
35	L12P	apolar → apolar			0.11	PI 475883	AD19
53	H18R	polar <sup>†</sup> → charged polar	1 AHASARQQWEL ARASARQQWEL		1.00	PI 475882 PI 475883	AD18 AD19
139	Q47E	polar → charged polar	4 LRPCEQHLMQ LRPCE <sup>E</sup> HLMQ	RRCQSQLERANLRPCEQHLM RRCQSQLERANLRPCE <sup>E</sup> HLM	1.00	PI 475882 PI 475883 PI 497270	AD18 AD19 AD29
218	S73T	polar → polar	7 SQDPYSPSY SQDPY <sup>T</sup> PSY		0.28	Grif 15035 Grif 15036	AD1 AD2
472	D158N	charged polar → polar			0.92	PI 468372 PI 497483	AD13 AD30

# Modeling Ara h 2



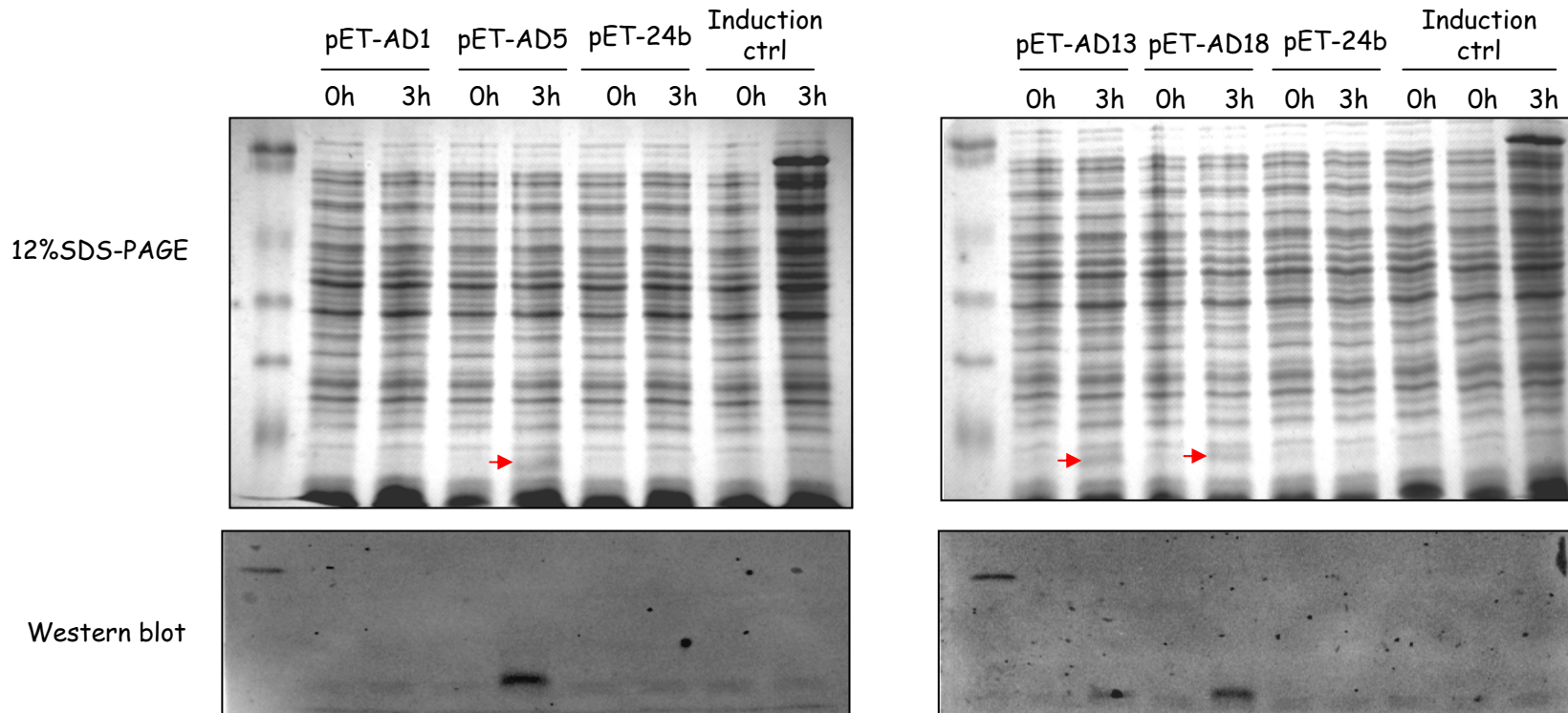


# Immunoblot (IgE Binding) and Densitometric Analysis of Accession Carrying S73T Mutation



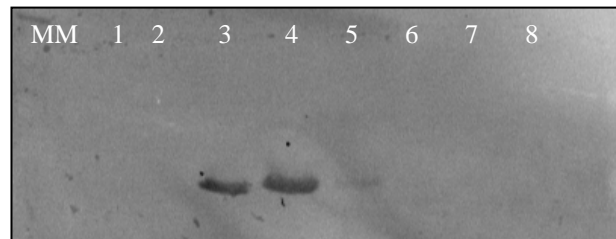
Reduction in IgE binding capacity  
56.2% (A), 98.93% (B) and 80.4%(C)

# Expression of recombinant proteins



## Purification of recombinant proteins

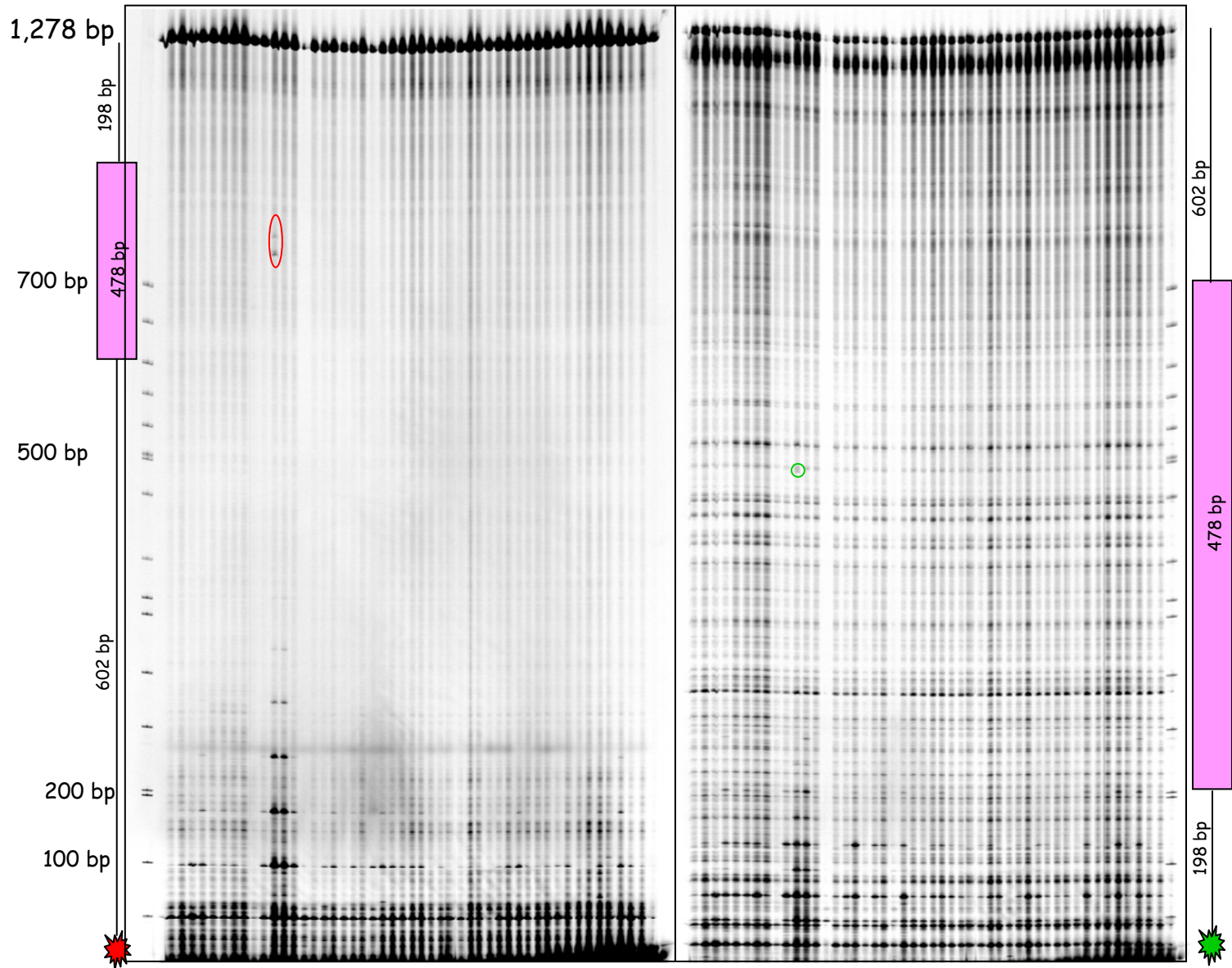
Immunoblot analysis of different aliquots from purification process of rAD5 (wt)



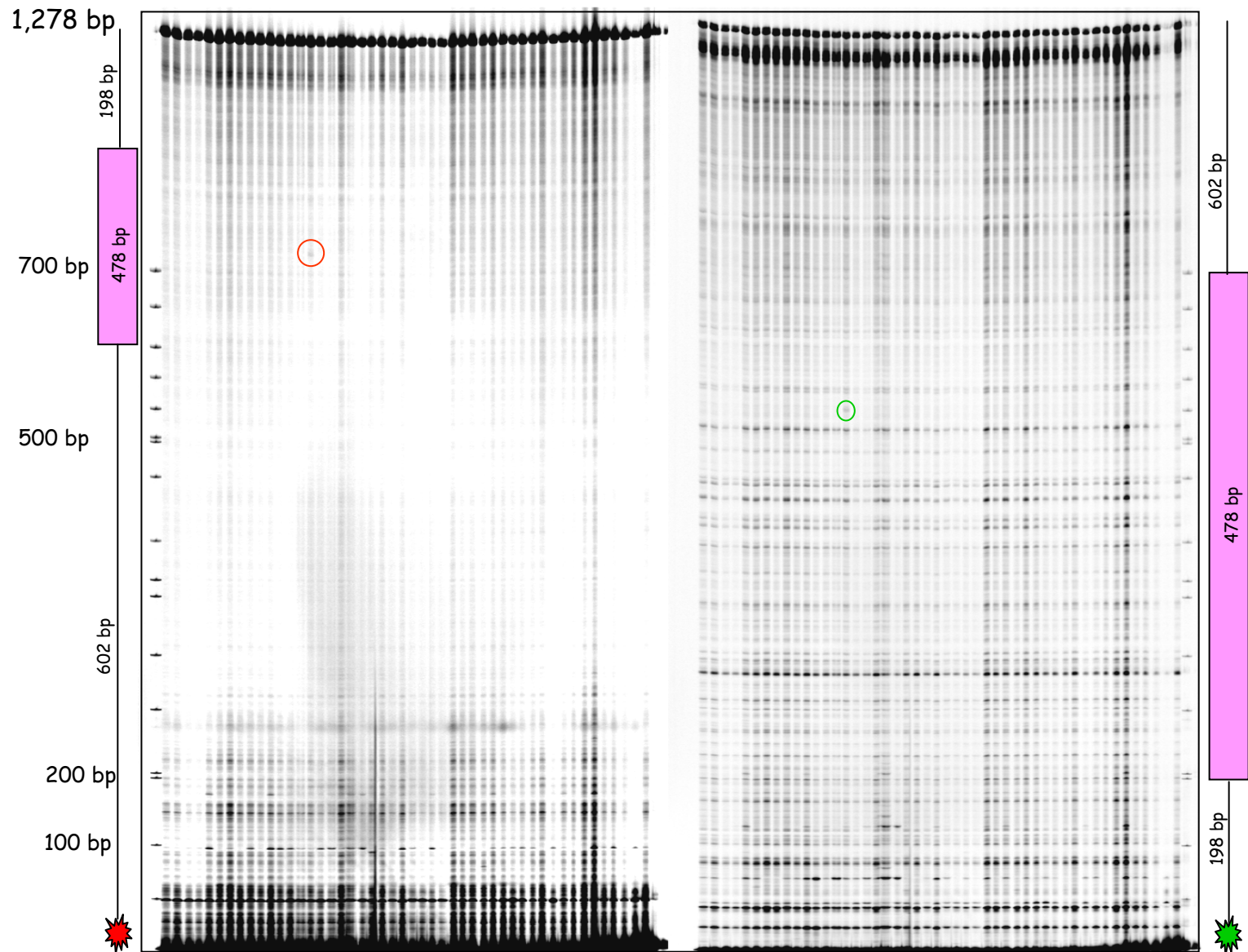
# Summary of Ecotilling

- Ecotilling allowed the identification of seven out of thirty *A. duranensis* accessions with putative mutations in the ORF of *ara d 2.01*
- The natural mutation rate found for this particular gene was 1 SNP/420 bp
- Missense and silent, but no non-sense mutations were detected
- Reduction in IgE binding capacity of 56.2% (A), 98.93% (B) and 80.4%(C) was observed for one isoform

# TILLING in EMS-mutagenized peanut population for *ara h 2.01* mutations

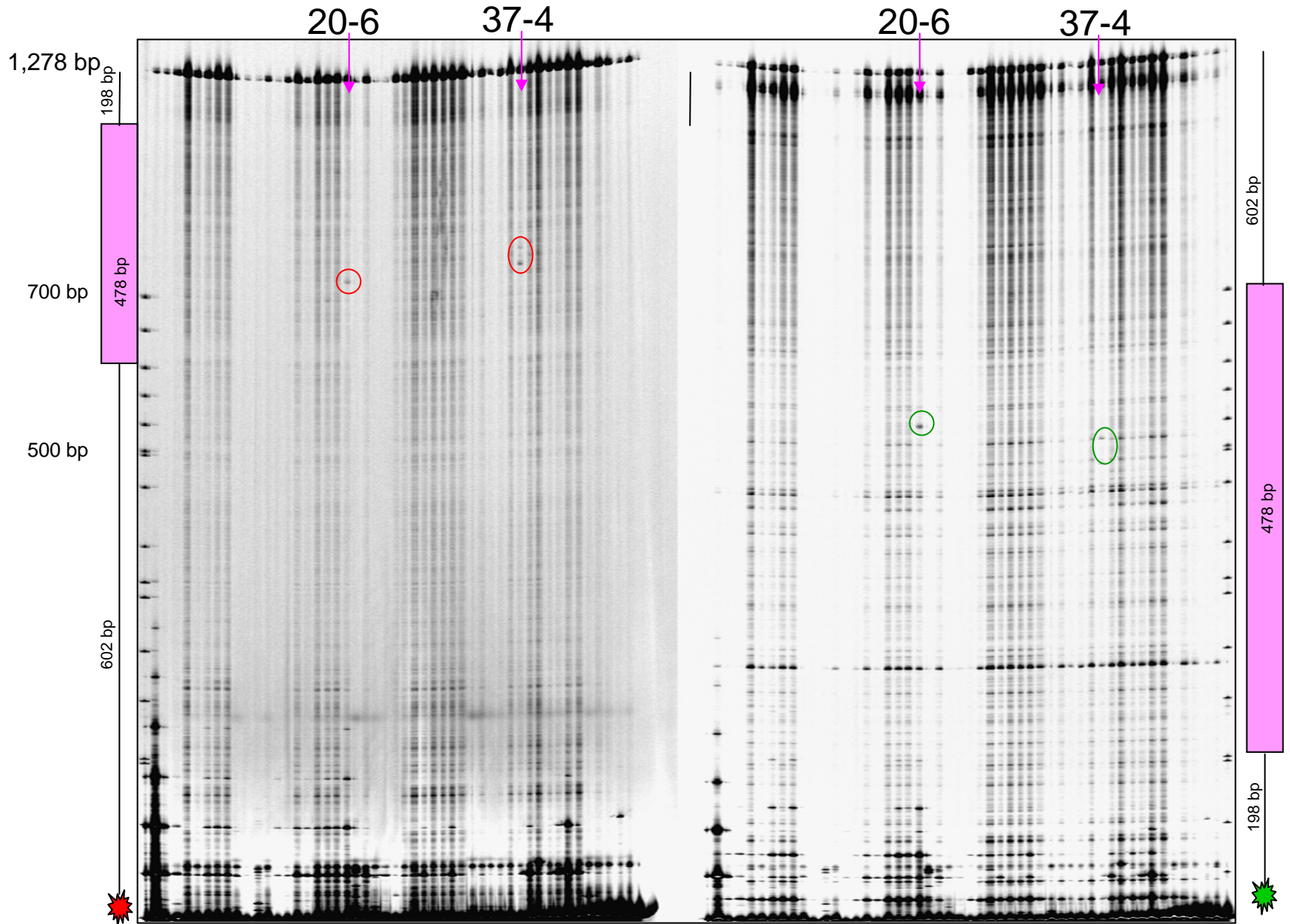


# *ara h 2.01*

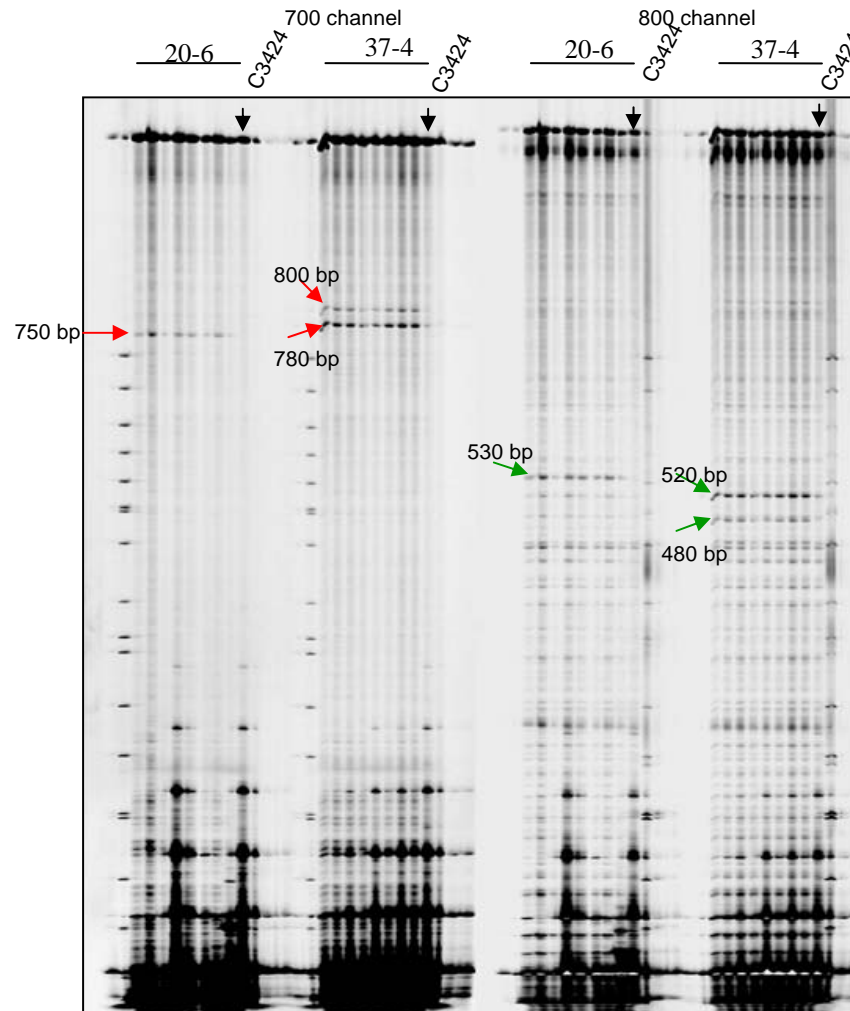




# Identification of individuals for *ara h 2.01*



# Detection of mutations in M3 generation of 20-6 and 37-4



[illegible]



# Conclusions of TILLING

- 2,020 individuals screened
- Recovered two validated *ara h 2.01* missense mutations and two putative mutants
- All mutants so far from EMS-mutagenized populations and for *ara h 2.01*
- Mutation rate 1 SNP/212 kb

# Silencing Allergens

## Silence by mutation (knockout)

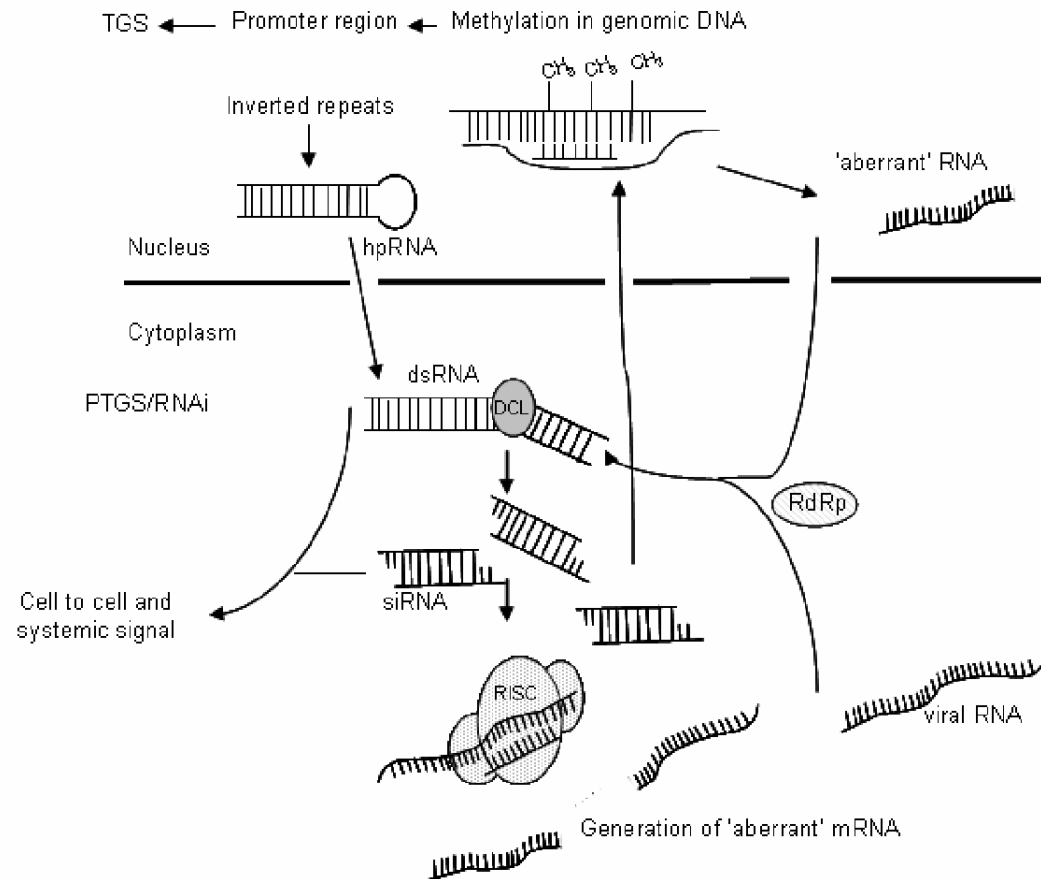
- Stable phenotype without genetic engineering
- Knockout is the less frequent mutation
- Only one gene targeted at one time

## Silence post-transcriptionally - at the level of expression (knockdown)

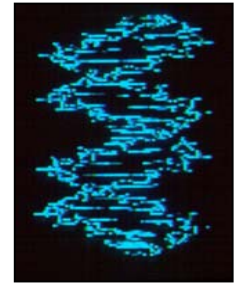
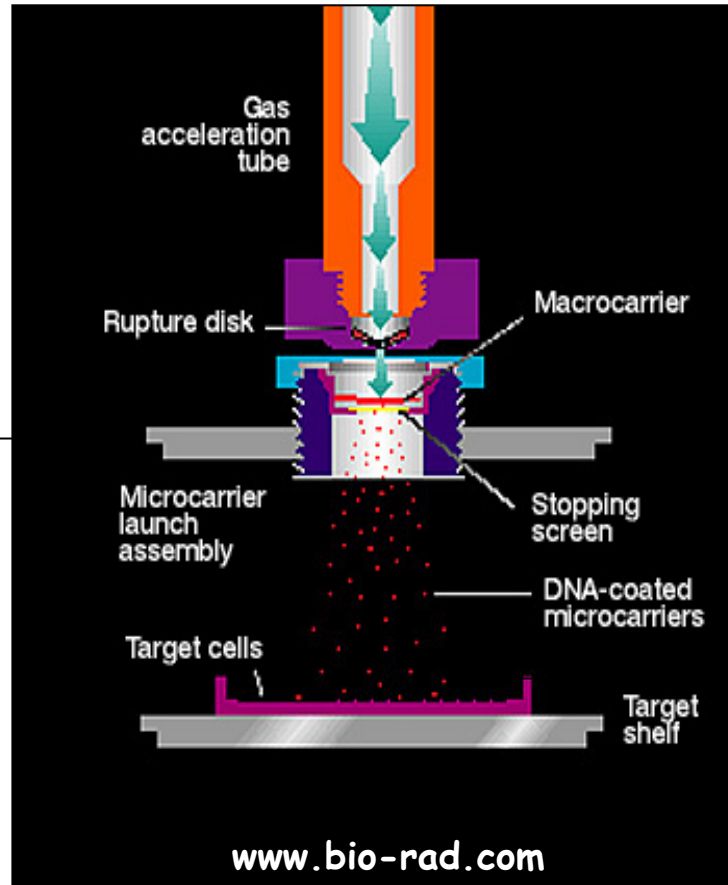
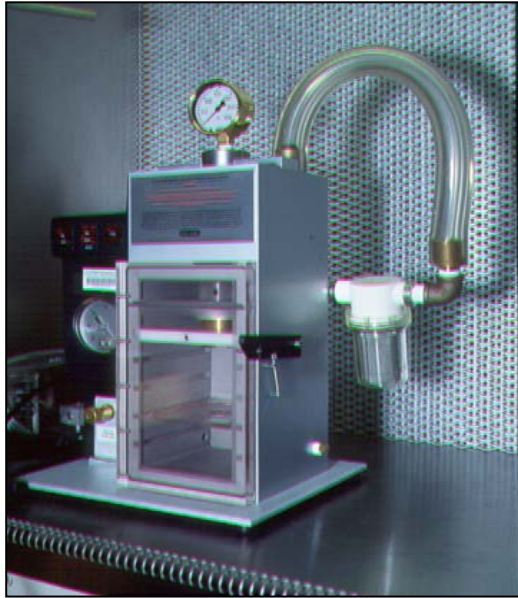
- Target multiple genes at one time
- Requires genetic engineering
- More variable in outcome and potentially unstable

# RNA Interference

Ozias-Akins *et al.*, 2006. Hypoallergenic foods beyond infant formulas.  
In: Food Allergy. ASM Press, Herndon, VA

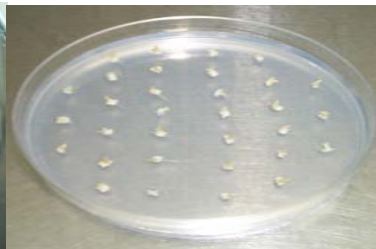


# Direct DNA Transformation

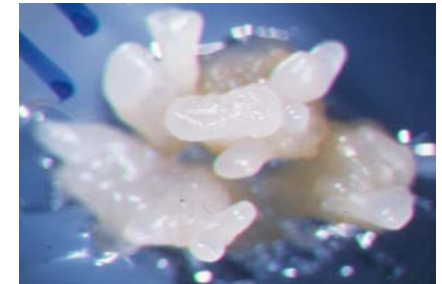


# TRANSFORMATION CYCLE

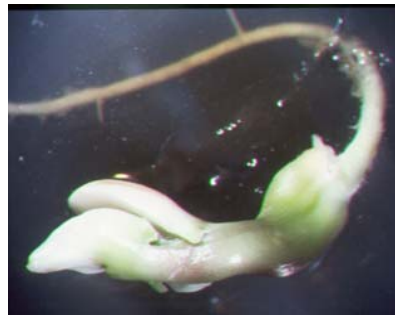
12-18 months



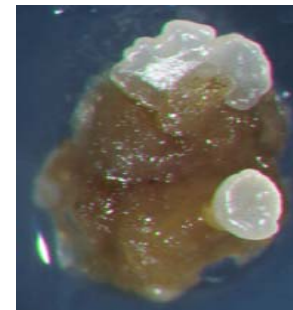
3-6  
months



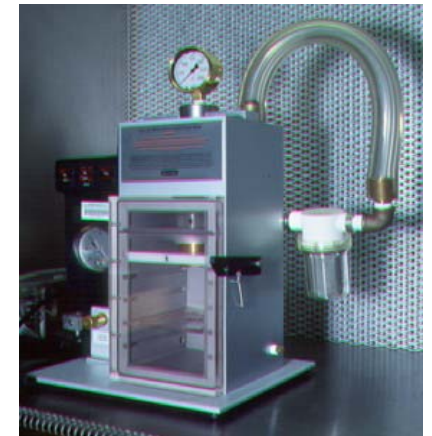
5 months



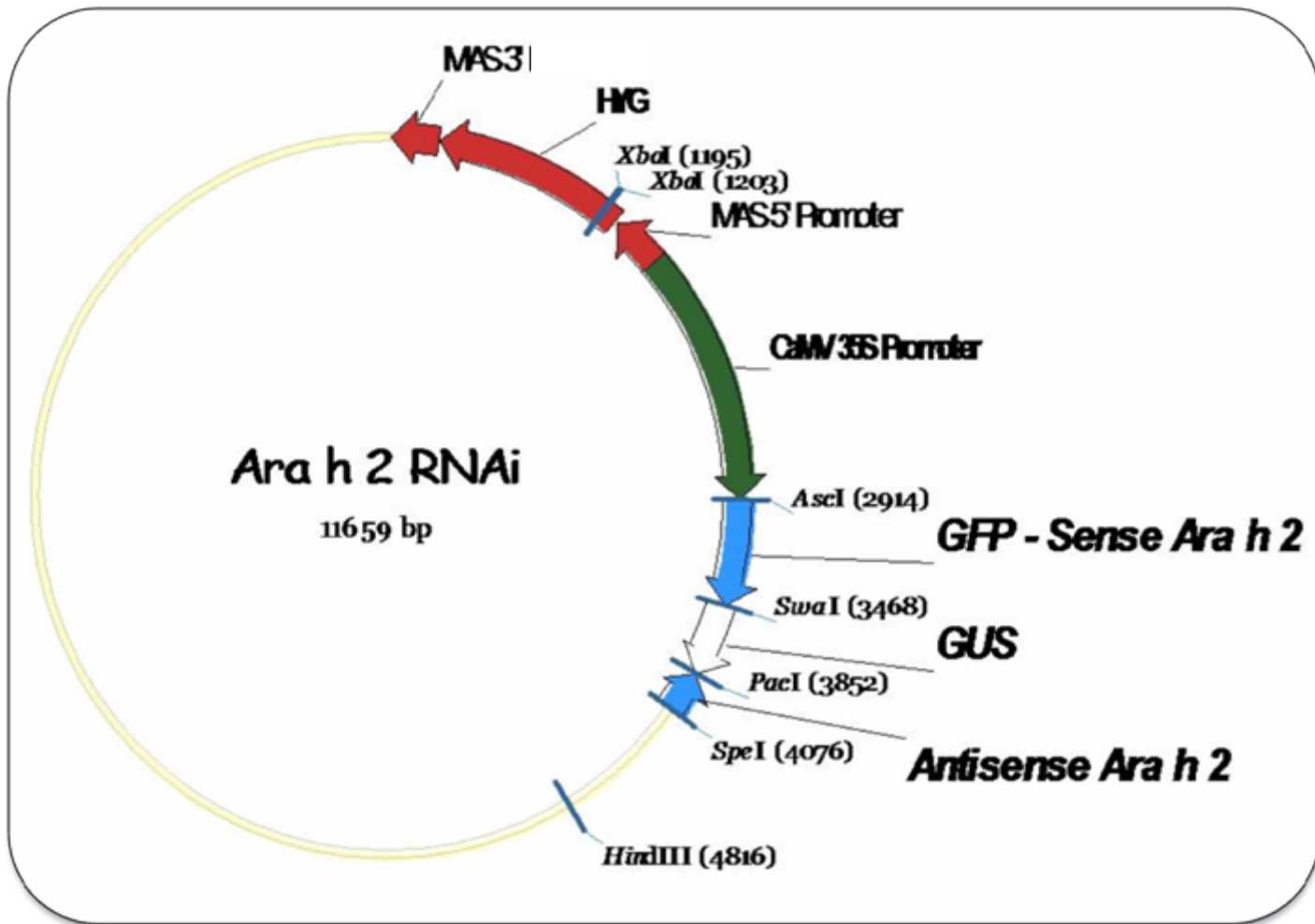
3 months



3 months



# Construct for RNA Interference of *ara h 2*





## GENETIC TRANSFORMATION

via biolistics



## SELECTION

Hygromycin  
resistant

8 hygromycin  
resistant lines

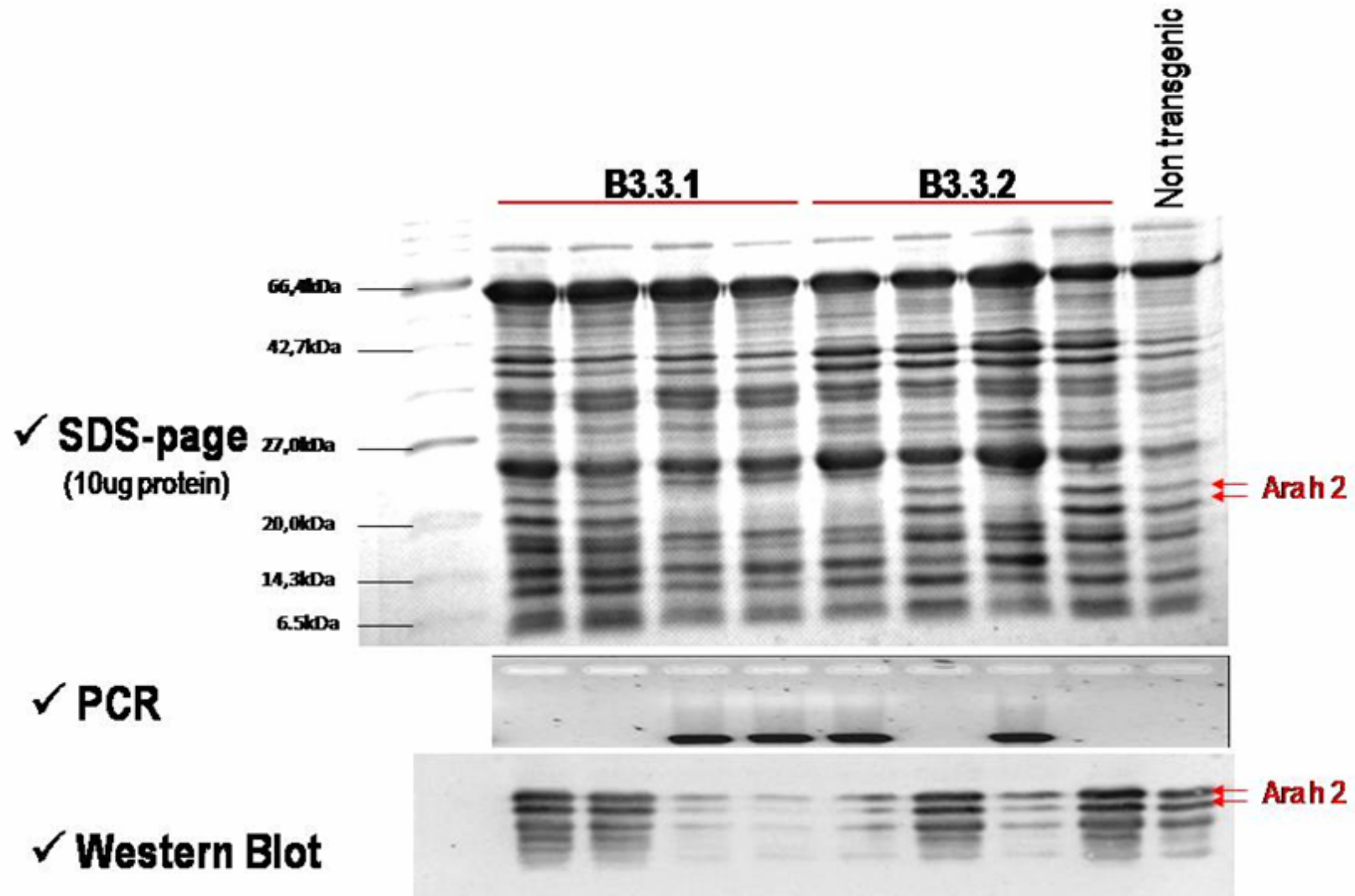


## TRANSGENIC LINES

- 4 lines: no sufficient seeds.
- 2 lines: B3.3.1 & B3.3.2  
reduced Ara h 2 protein
- 2 lines: B4 & B11  
barely detectable Ara h 2.



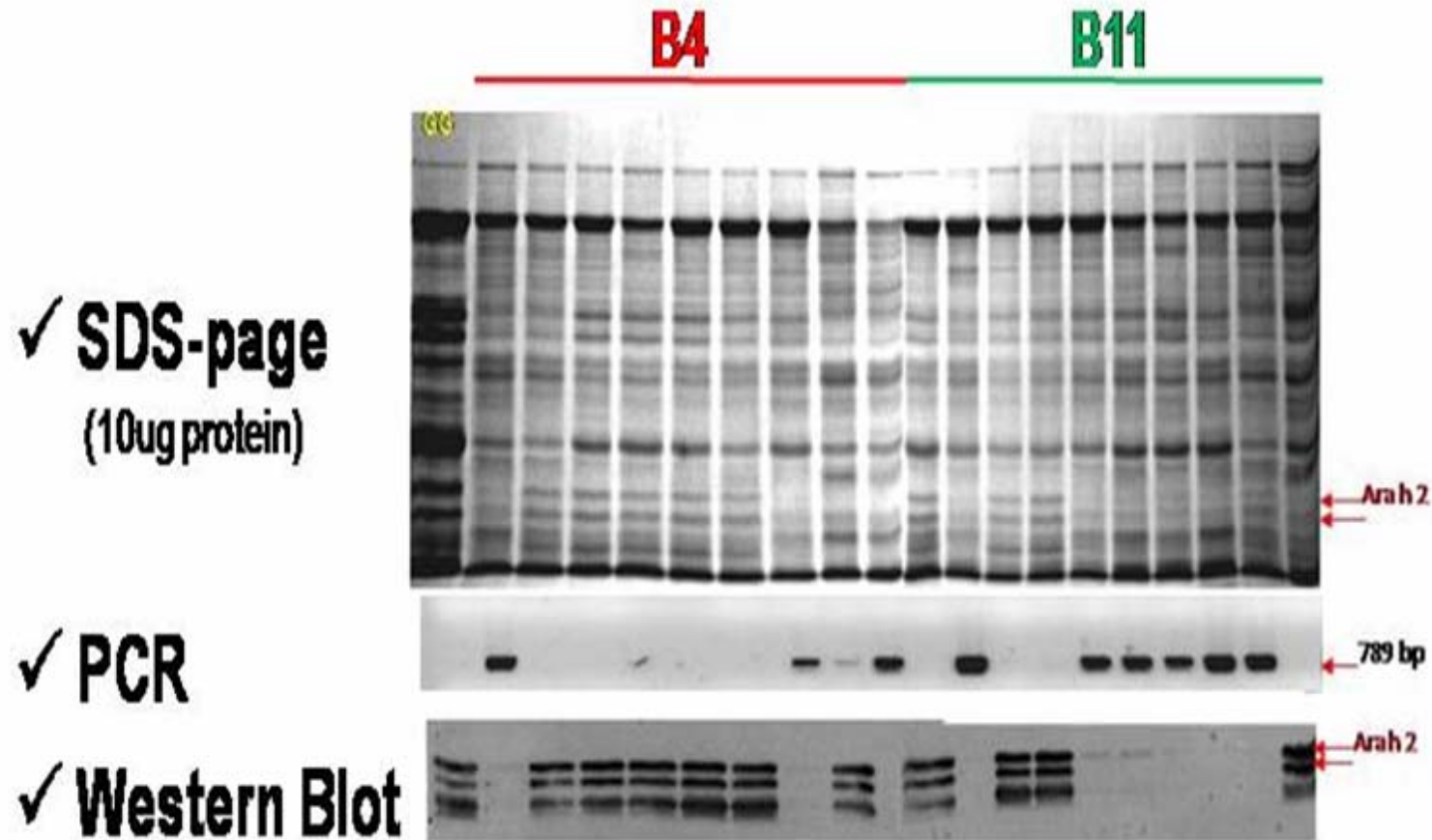
Ara h 2 is reduced by ~90% in some transgenic lines



(1<sup>st</sup> anti-Ara h 2 antibody from chicken; 2<sup>nd</sup> Anti-chicken conjugated with horseradish peroxidase - 10ug protein)



Ara h 2 is barely detectable in other transgenic lines

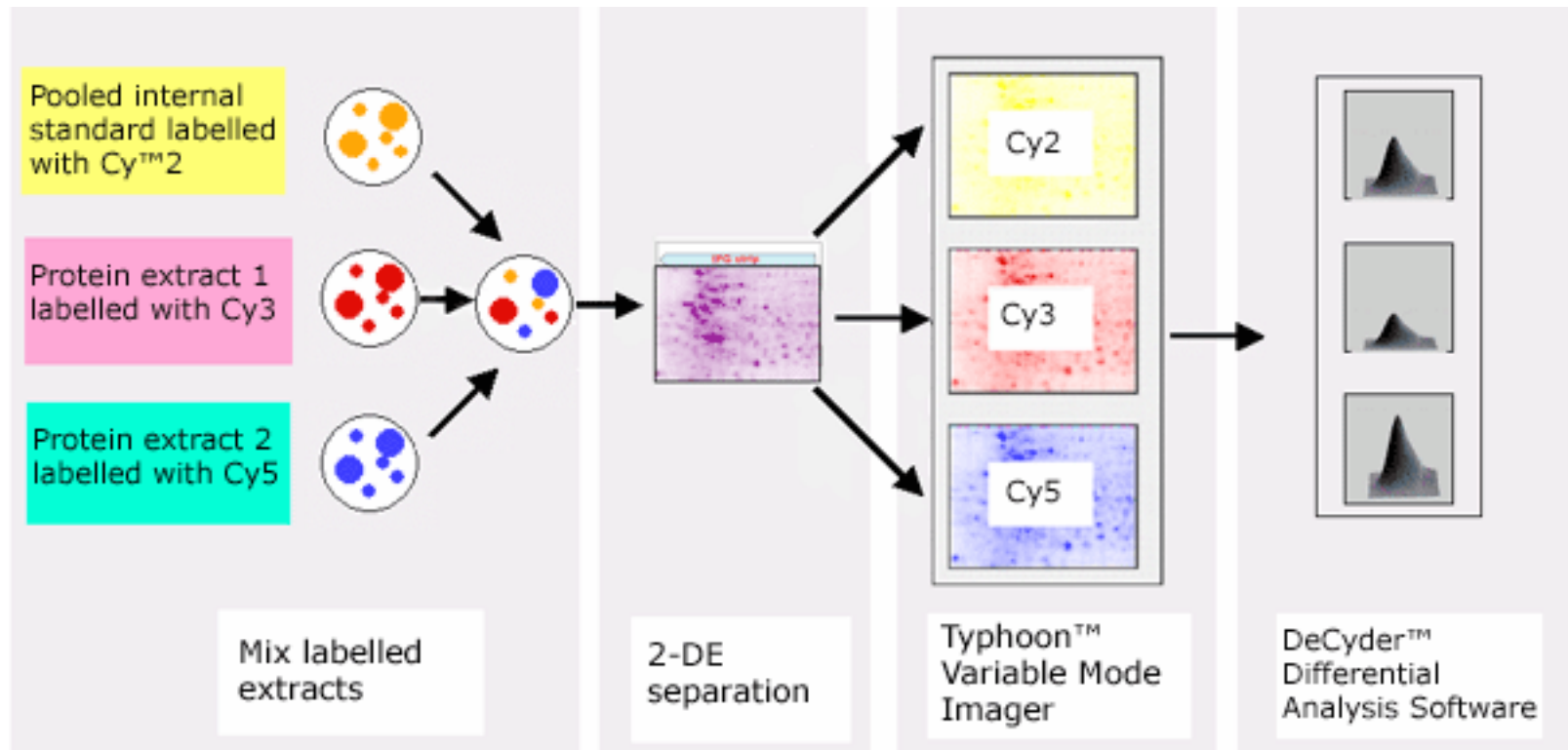


(1<sup>st</sup> anti-Ara h 2 antibody from chicken; 2<sup>nd</sup> Anti-chicken conjugated with horseradish peroxidase  
- 10ug protein)

# New variation of 2-D gels...Difference Gel Electrophoresis (DIGE)

## Differential Gel Electrophoresis (DIGE)

- samples resolved in the same gel
- simplifies spot matching

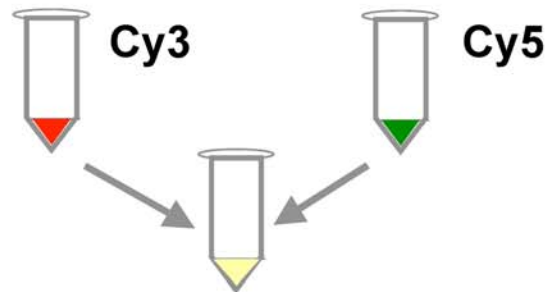


# Difference Gel Electrophoresis - DIGE

Sample 1

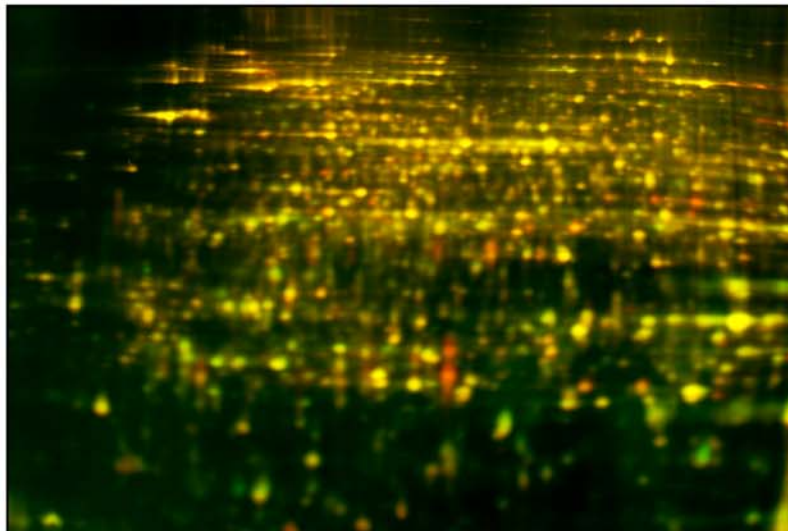
Sample 2

50  $\mu$ g protein



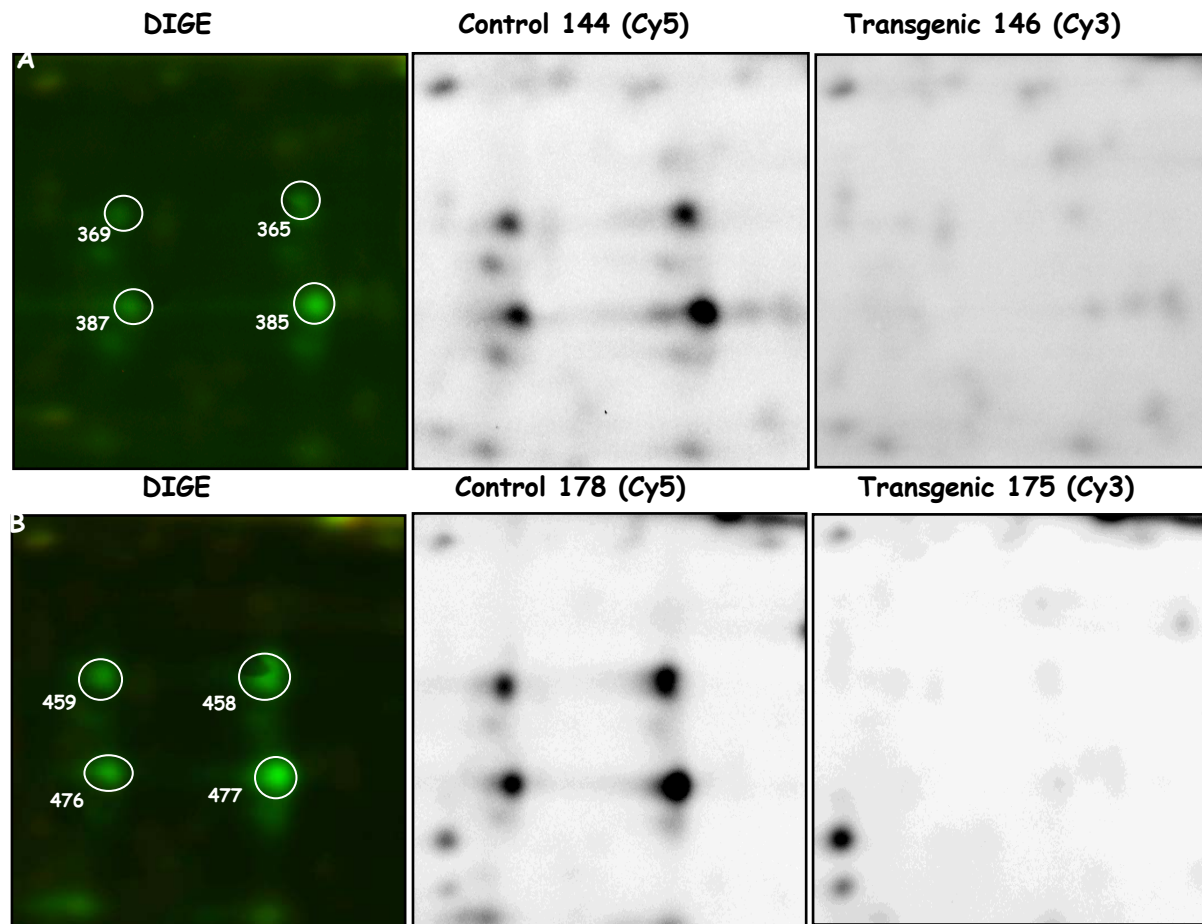
Cyanine dye label

- Lys reactive
- charge balanced
- 0.2 ng sensitivity

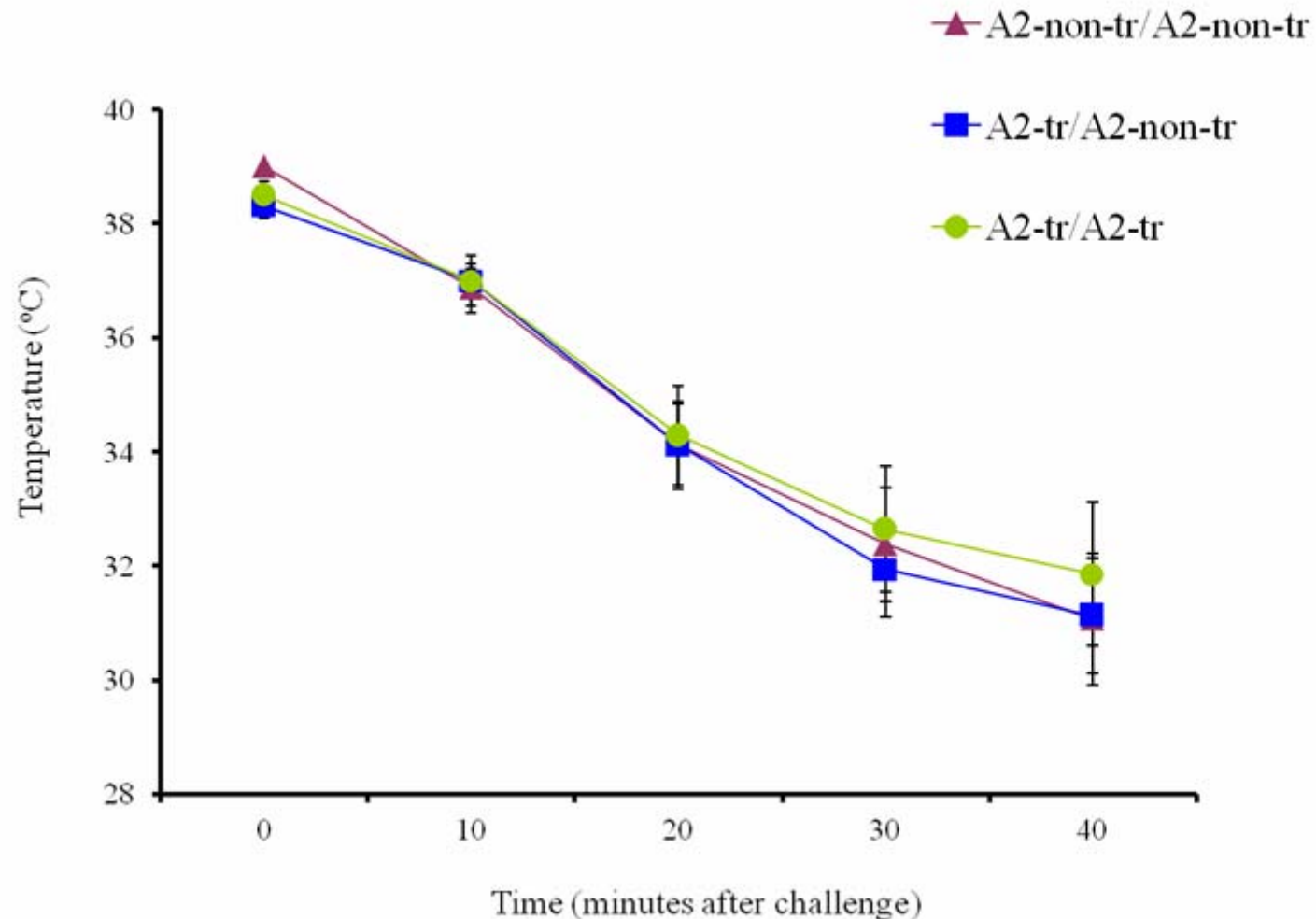


Arabidopsis seed

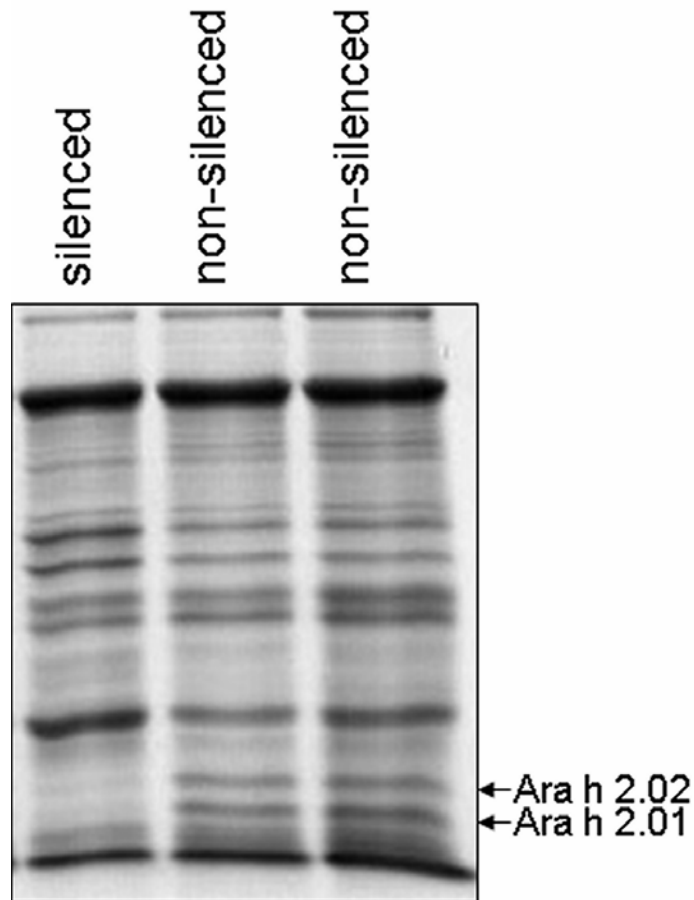
# Difference Gel Electrophoretic Analysis Coupled with Tandem Mass Spectrometry



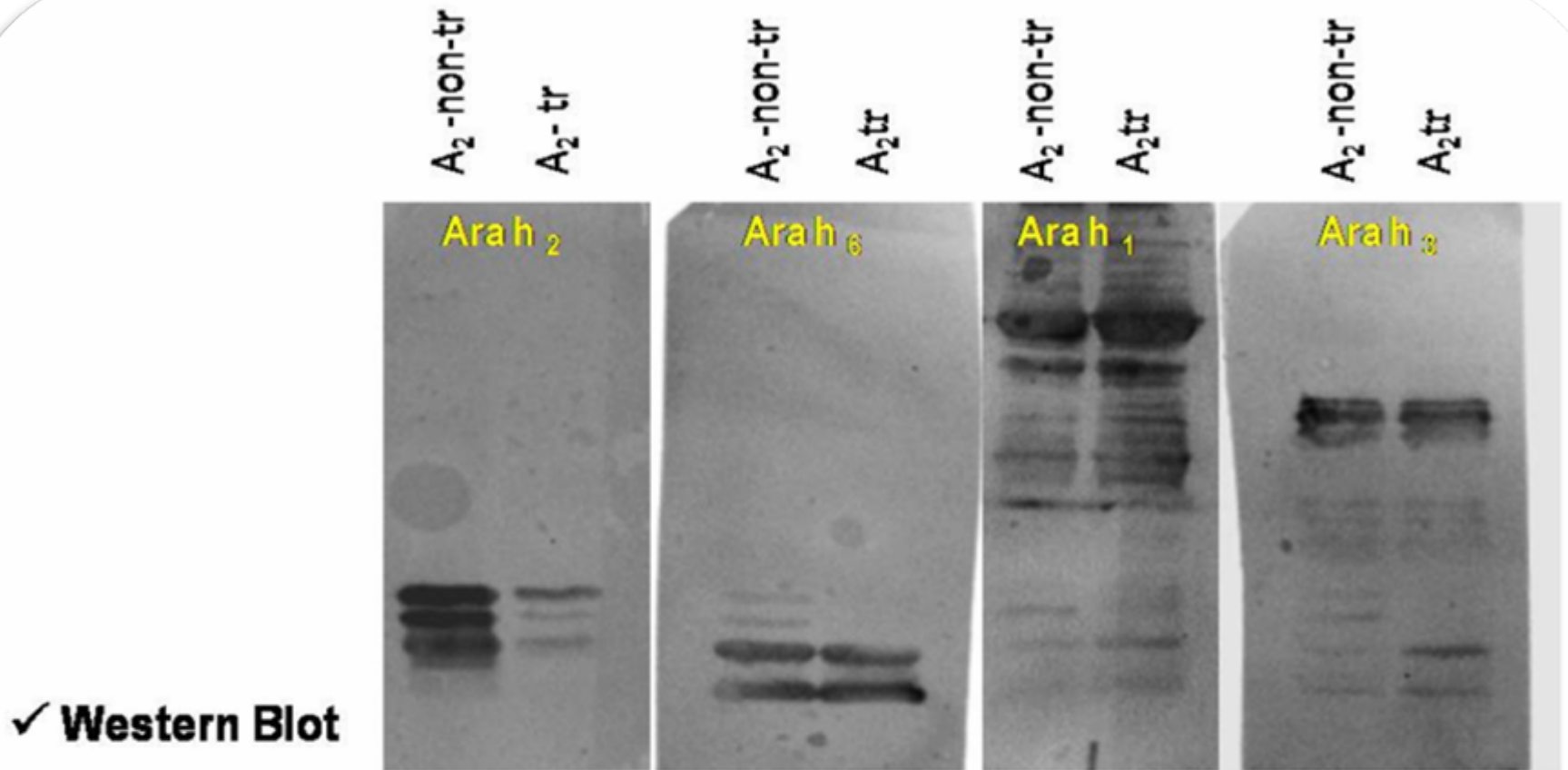
# A 90% reduction in Ara h 2 is not sufficient to alter allergic reaction in a mouse model of anaphylaxis



# RNA Interference Is Specific to Ara h 2 . . .



# RNA Interference Is Specific to Ara h 2 . . .

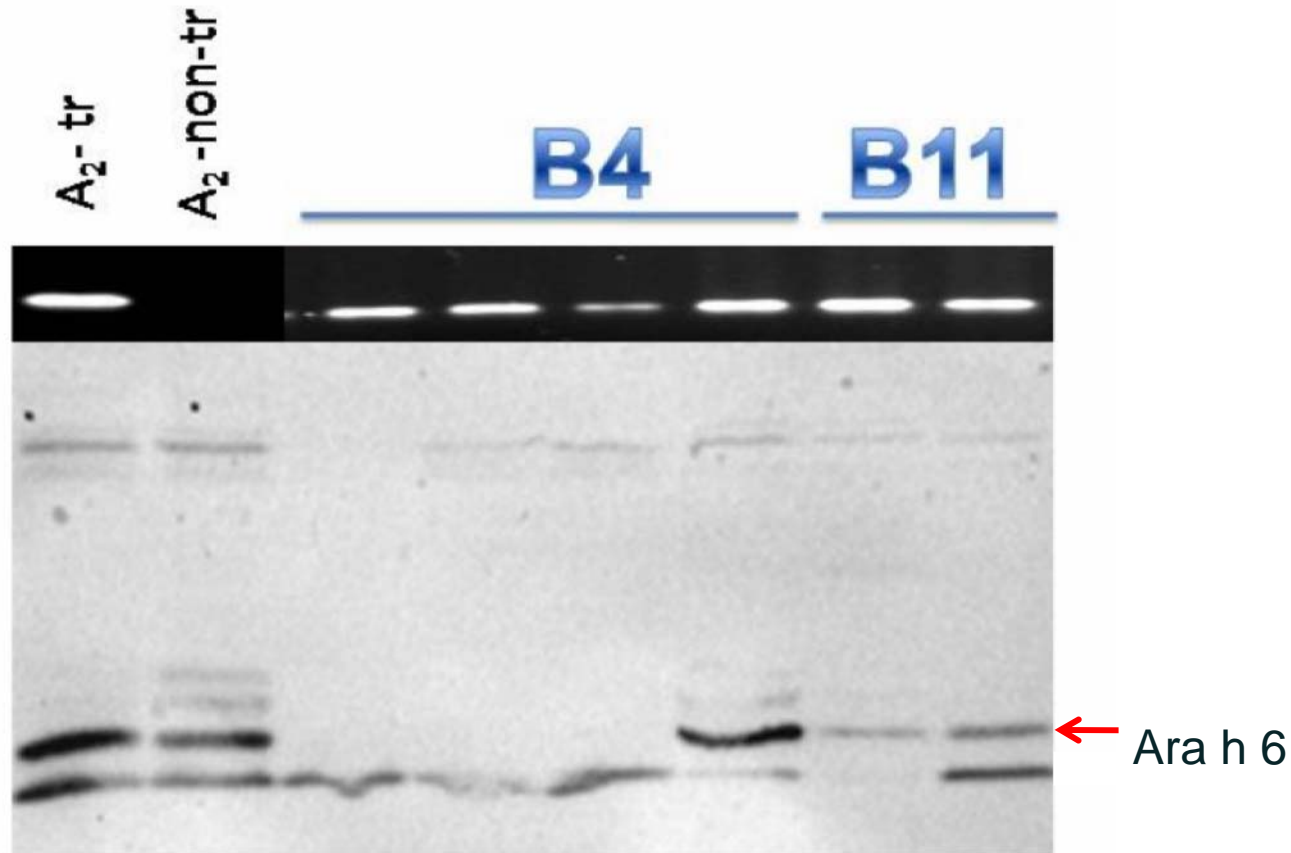


✓ Western Blot

(1<sup>o</sup> anti-Ara h 2, Ara h 6, Ara h 1 and Ara h 3 antibody from chicken; 2<sup>o</sup> Anti-chicken conjugated with horseradish peroxidase - 10ug protein)



# RNA Interference Is Specific to Ara h 2 Except for Ara h 6 in Some Lines





# Conclusions

- ❖ RNA silencing is an effective method to silence allergen genes at least for the purpose of testing the effect of their absence on seed growth and development.
- ❖ RNA silencing may be unstable under certain circumstances, and instability cannot be tolerated in a commercial crop.
- ❖ TILLING to recover mutant allergen genes is likely to result in more stable reduction of allergens, but will require many steps including mutant production, mutant screening, and traditional breeding to combine mutant A- and B-genome genes.

# Acknowledgements

Consortium for Plant Biotechnology Research

Georgia Peanut Commission

Peanut Foundation

National Peanut Board

USDA

Evelyn Morgan and Anne Bell