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GENEVA

WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA PROFILING IN PARTICULAR

Eleventh Session
Madrid, September 16 to 18, 2008

ADDENDUM

COMPARATIVE GENOMIC HYBRIDIZATION FOR IDENTIFYING MUTATION VARIETIES

Document prepared by experts from Spain

Comparative Genomic Hybridization for identifying mutation varieties in citrus

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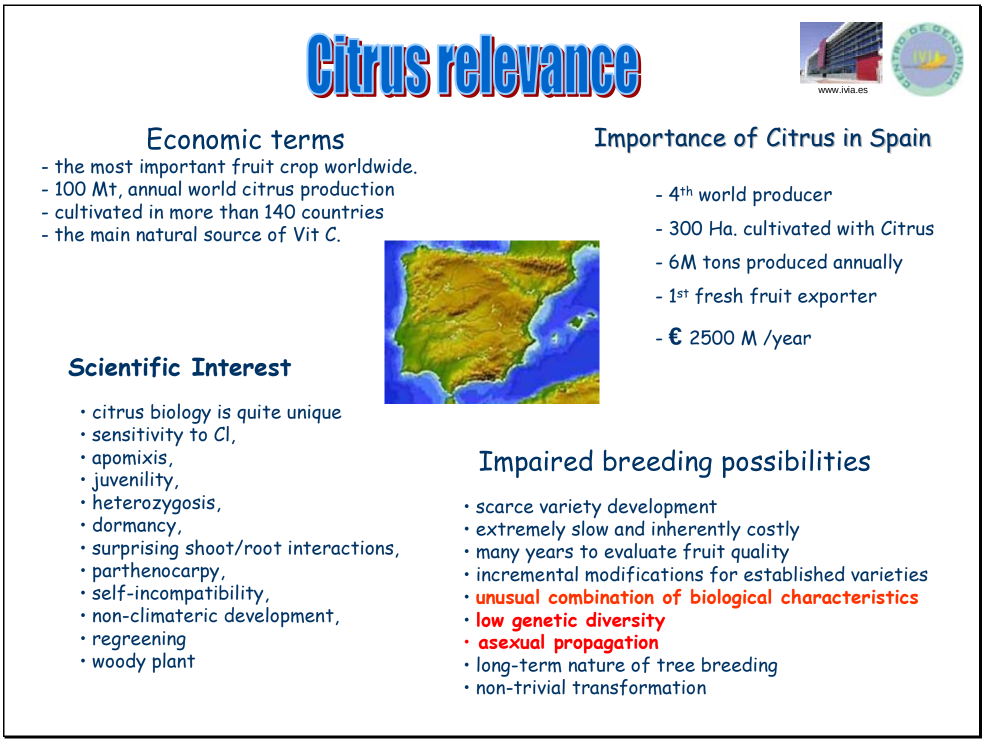


Citrus relevance

www.ivia.es

Economic terms

- the most important fruit crop worldwide.
- 100 Mt, annual world citrus production
- cultivated in more than 140 countries
- the main natural source of Vit C.

Importance of Citrus in Spain

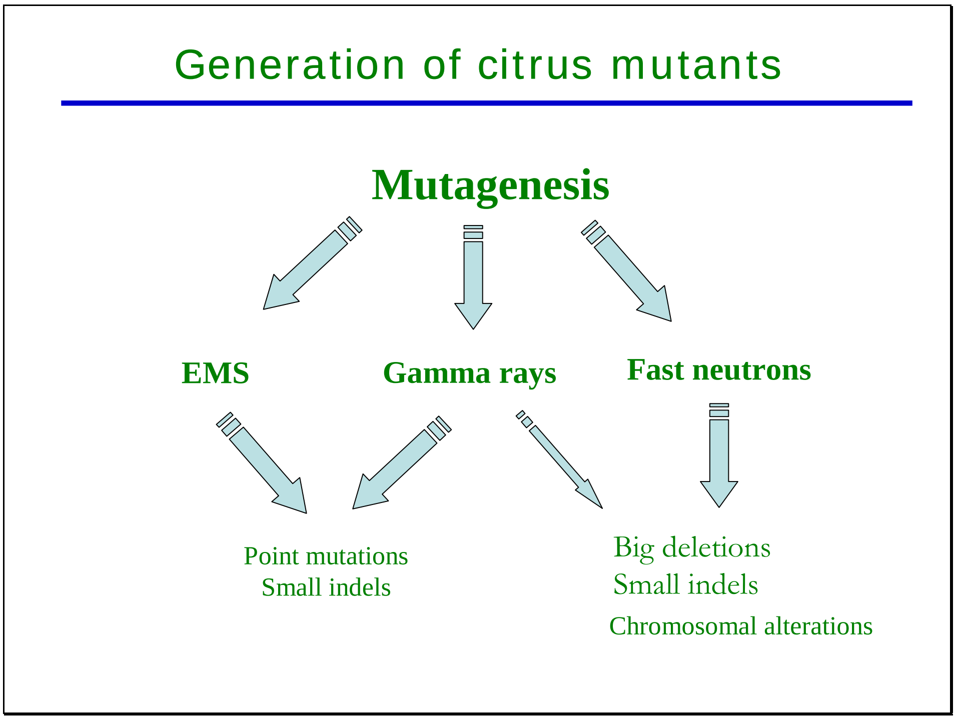
- 4th world producer
- 300 Ha. cultivated with Citrus
- 6M tons produced annually
- 1st fresh fruit exporter
- € 2500 M /year

Scientific Interest

- citrus biology is quite unique
- sensitivity to Cl,
- apomixis,
- juvenility,
- heterozygosis,
- dormancy,
- surprising shoot/root interactions,
- parthenocarpy,
- self-incompatibility,
- non-climateric development,
- regreening
- woody plant

Impaired breeding possibilities

- scarce variety development
- extremely slow and inherently costly
- many years to evaluate fruit quality
- incremental modifications for established varieties
- **unusual combination of biological characteristics**
- **low genetic diversity**
- **asexual propagation**
- long-term nature of tree breeding
- non-trivial transformation



Generation of citrus mutants

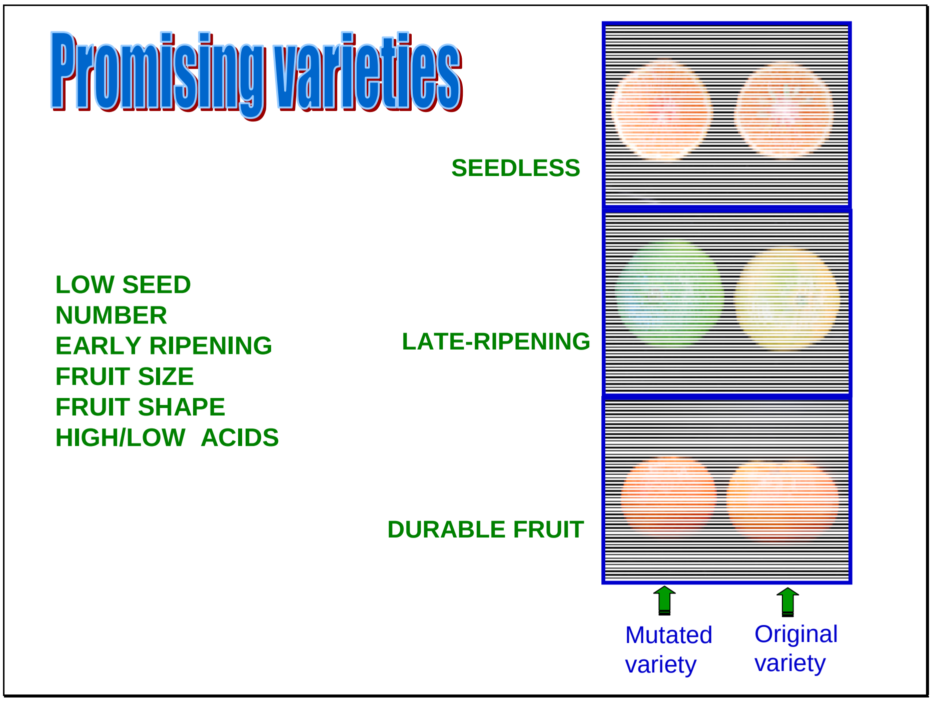
Mutagenesis

EMS **Gamma rays** **Fast neutrons**

Point mutations
Small indels

Big deletions
Small indels
Chromosomal alterations

Citrus Mutants



Promising varieties

SEEDLESS

LOW SEED NUMBER
EARLY RIPENING
FRUIT SIZE
FRUIT SHAPE
HIGH/LOW ACIDS

LATE-RIPENING

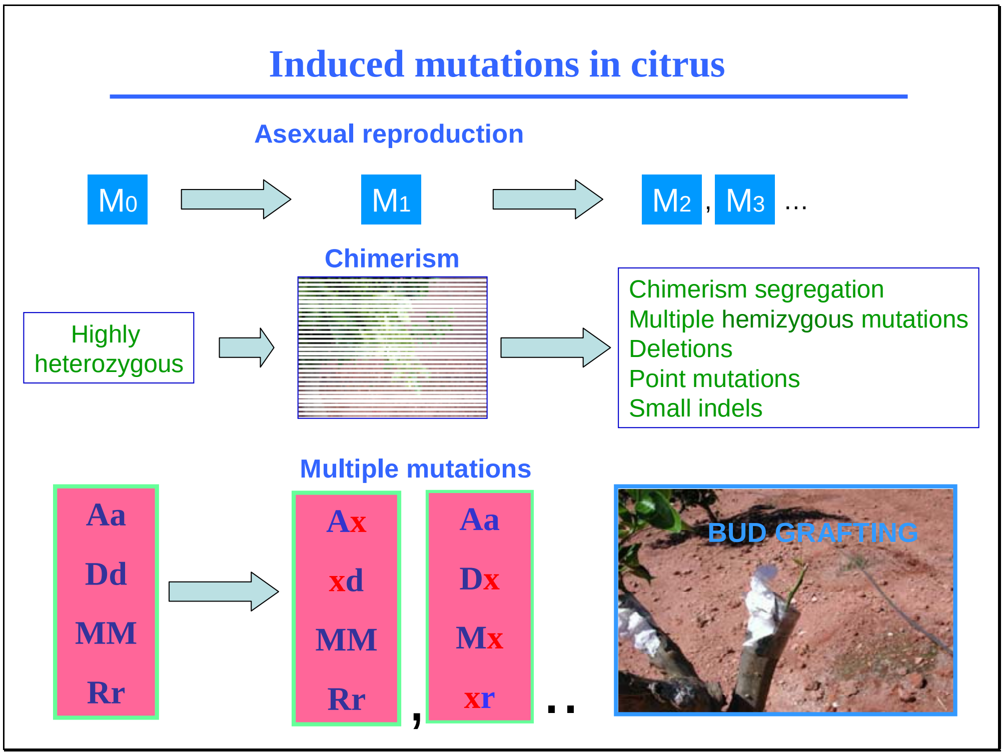
DURABLE FRUIT

Mutated variety

Original variety

Identification of Mutation Varieties

- Can we distinguish them at the molecular level?
- Can we provide a method for unequivocal identification?
- How can these relate to the parental varieties?
- What specific changes have been introduced in the genome of the mutated varieties?



Induced mutations in citrus

Asexual reproduction

M0 → M1 → M2 , M3 ...

Chimerism

Highly heterozygous → →

Chimerism segregation
Multiple hemizygous mutations
Deletions
Point mutations
Small indels

Multiple mutations

Aa
Dd
MM
Rr

→

Ax
xd
MM
Rr
,
Aa
Dx
Mx
xr
. .

Can we characterize these hemyzigous mutations ?

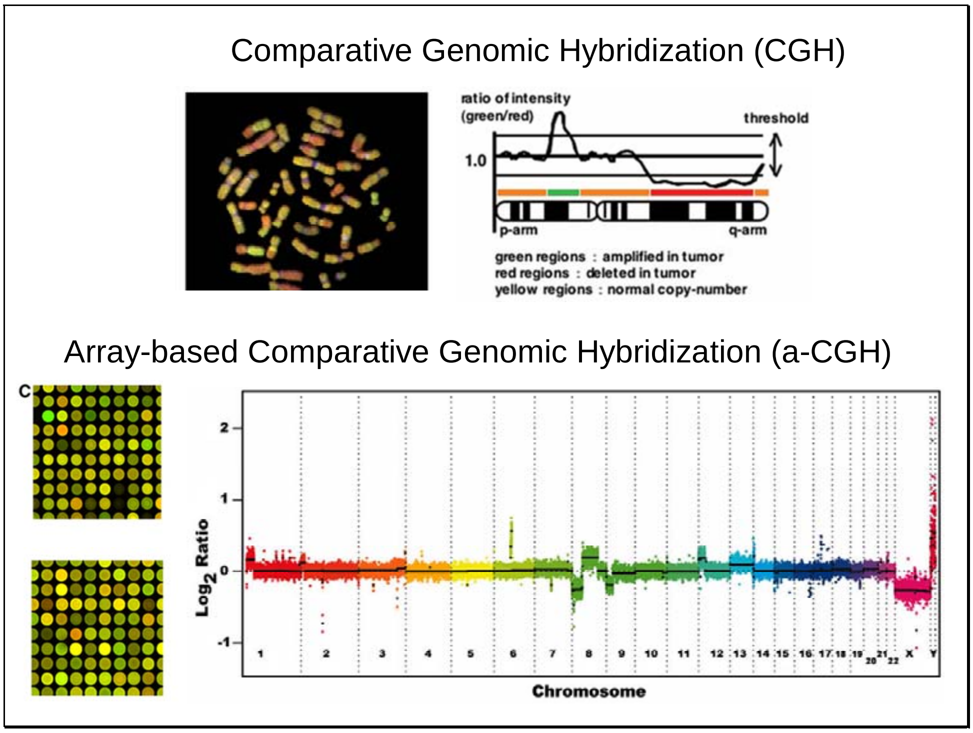
POINT MUTATIONS

- -Expression profiling
- -Reverse Genetics. Tilling
- -DHPLC (Denaturing HPLC)
- -Oligonucleotide array

DELETIONS

- -Genomic subtraction
- -Expression profiling
- -Comparative genomic hybridization

Identification of deleted genes
through
Comparative Genomic Hybridization
using microarrays

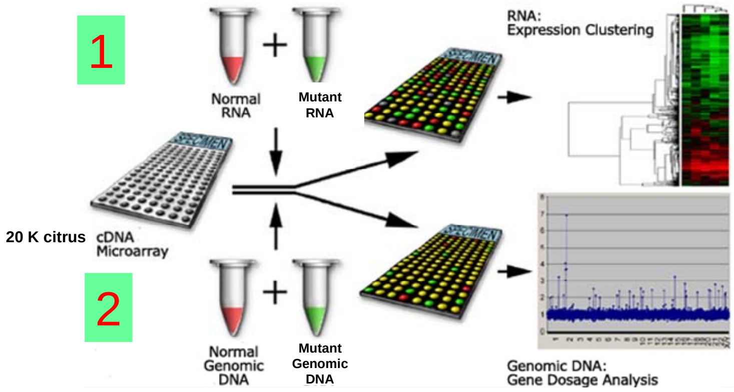


Comparative Genomic Hybridization (CGH)

Array-based Comparative Genomic Hybridization (a-CGH)

Spanish Citrus Genomics Consortium

Microarrays



1

Normal RNA + Mutant RNA

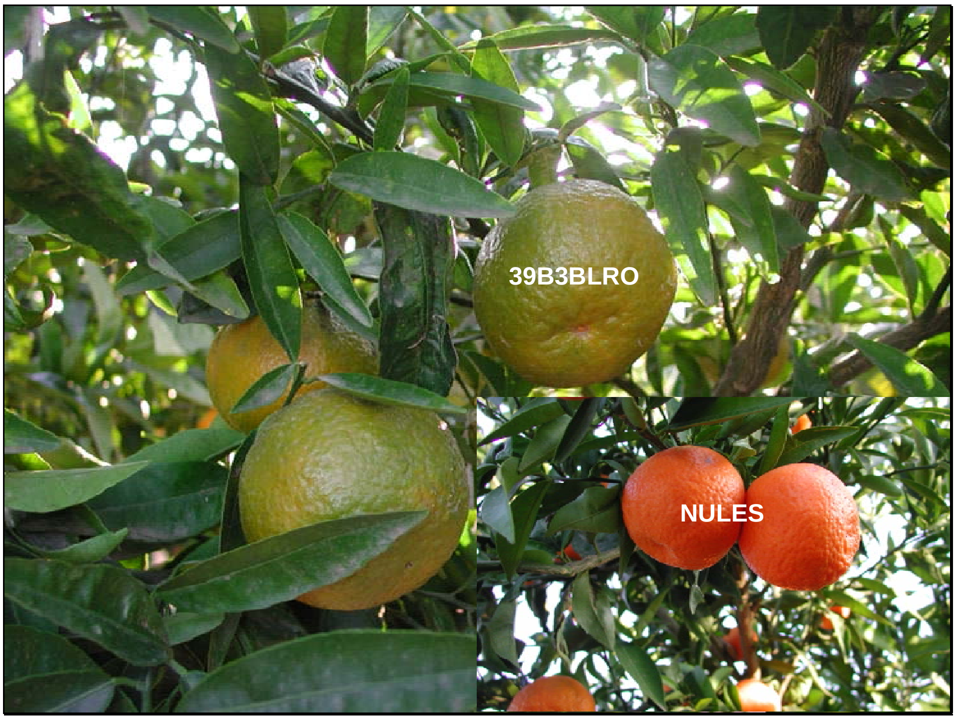
20 K citrus cDNA Microarray

RNA: Expression Clustering

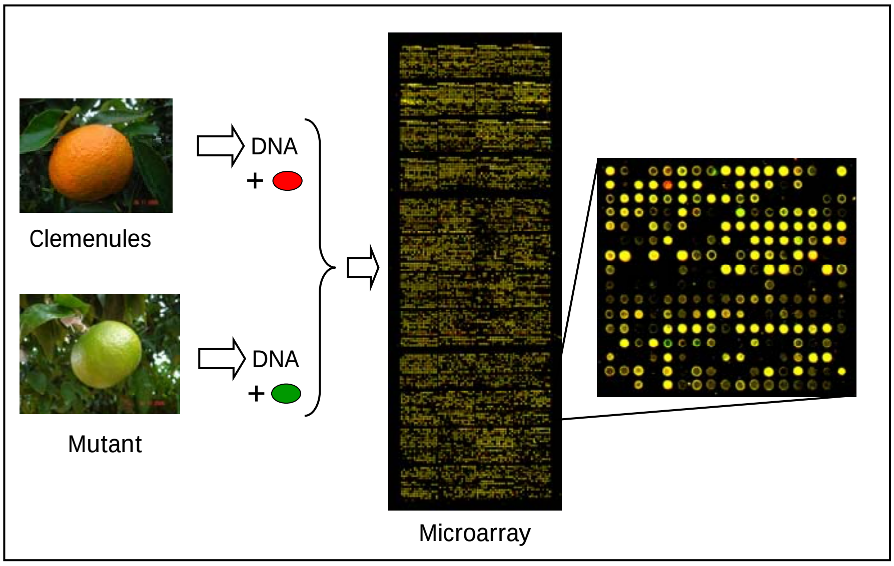
2

Normal Genomic DNA + Mutant Genomic DNA

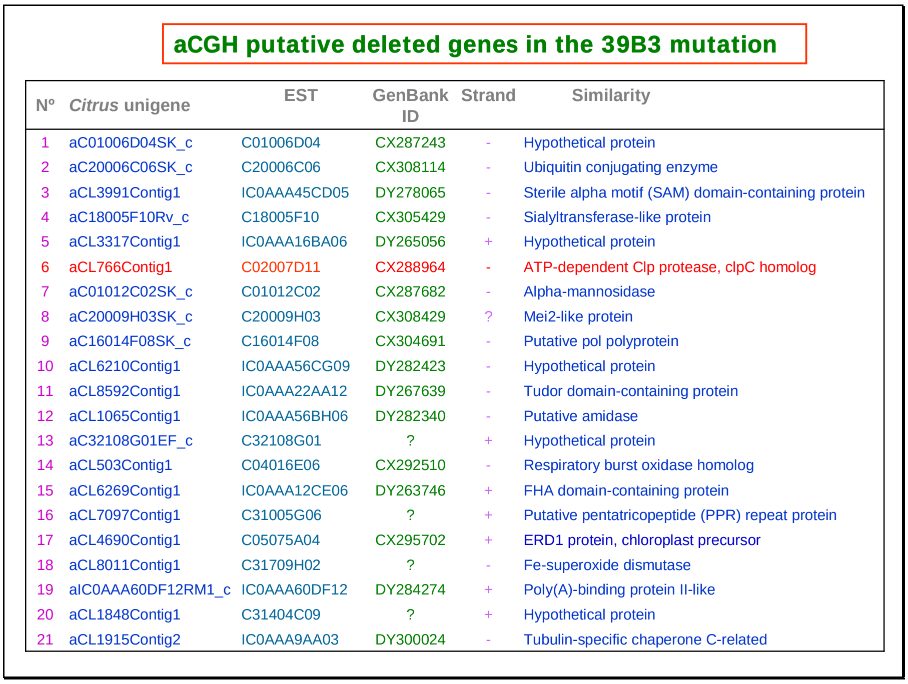
Genomic DNA: Gene Dosage Analysis



Microarray-based CGH

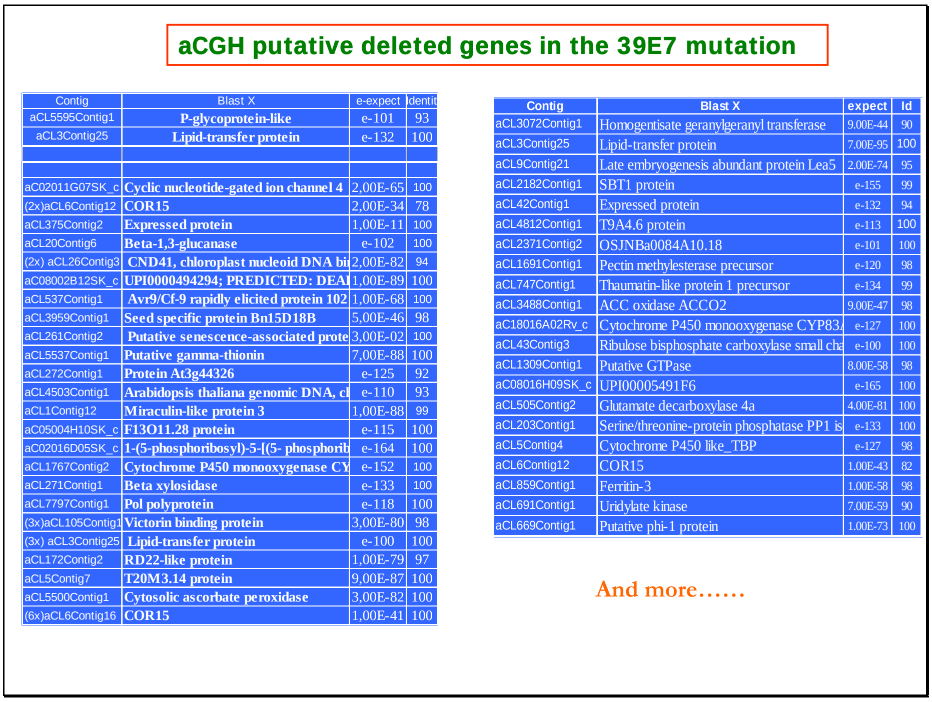


-21 putative deleted genes in 39B3
-78 putative deleted genes in 39E7



aCGH putative deleted genes in the 39B3 mutation

Nº	*Citrus* unigene	EST	GenBank ID	Strand	Similarity
1	aC01006D04SK_c	C01006D04	CX287243	-	Hypothetical protein
2	aC20006C06SK_c	C20006C06	CX308114	-	Ubiquitin conjugating enzyme
3	aCL3991Contig1	IC0AAA45CD05	DY278065	-	Sterile alpha motif (SAM) domain-containing protein
4	aC18005F10Rv_c	C18005F10	CX305429	-	Sialyltransferase-like protein
5	aCL3317Contig1	IC0AAA16BA06	DY265056	+	Hypothetical protein
6	aCL766Contig1	C02007D11	CX288964	-	ATP-dependent Clp protease, clpC homolog
7	aC01012C02SK_c	C01012C02	CX287682	-	Alpha-mannosidase
8	aC20009H03SK_c	C20009H03	CX308429	?	Mei2-like protein
9	aC16014F08SK_c	C16014F08	CX304691	-	Putative pol polyprotein
10	aCL6210Contig1	IC0AAA56CG09	DY282423	-	Hypothetical protein
11	aCL8592Contig1	IC0AAA22AA12	DY267639	-	Tudor domain-containing protein
12	aCL1065Contig1	IC0AAA56BH06	DY282340	-	Putative amidase
13	aC32108G01EF_c	C32108G01	?	+	Hypothetical protein
14	aCL503Contig1	C04016E06	CX292510	-	Respiratory burst oxidase homolog
15	aCL6269Contig1	IC0AAA12CE06	DY263746	+	FHA domain-containing protein
16	aCL7097Contig1	C31005G06	?	+	Putative pentatricopeptide (PPR) repeat protein
17	aCL4690Contig1	C05075A04	CX295702	+	ERD1 protein, chloroplast precursor
18	aCL8011Contig1	C31709H02	?	-	Fe-superoxide dismutase
19	aIC0AAA60DF12RM1_c	IC0AAA60DF12	DY284274	+	Poly(A)-binding protein II-like
20	aCL1848Contig1	C31404C09	?	+	Hypothetical protein
21	aCL1915Contig2	IC0AAA9AA03	DY300024	-	Tubulin-specific chaperone C-related



aCGH putative deleted genes in the 39E7 mutation

Contig	Blast X	e-expect	Identit
aCL5595Contig1	**P-glycoprotein-like**	e-101	93
aCL3Contig25	**Lipid-transfer protein**	e-132	100
aC02011G07SK_c	**Cyclic nucleotide-gated ion channel 4**	2,00E-65	100
(2x)aCL6Contig12	**COR15**	2,00E-34	78
aCL375Contig2	**Expressed protein**	1,00E-11	100
aCL20Contig6	**Beta-1,3-glucanase**	e-102	100
(2x) aCL26Contig3	**CND41, chloroplast nucleoid DNA bi**	2,00E-82	94
aC08002B12SK_c	**UPI0000494294; PREDICTED: DEA**	1,00E-89	100
aCL537Contig1	**Avr9/Cf-9 rapidly elicited protein 102**	1,00E-68	100
aCL3959Contig1	**Seed specific protein Bn15D18B**	5,00E-46	98
aCL261Contig2	**Putative senescence-associated prote**	3,00E-02	100
aCL5537Contig1	**Putative gamma-thionin**	7,00E-88	100
aCL272Contig1	**Protein At3g44326**	e-125	92
aCL4503Contig1	**Arabidopsis thaliana genomic DNA, c**	e-110	93
aCL1Contig12	**Miraculin-like protein 3**	1,00E-88	99
aC05004H10SK_c	**F13O11.28 protein**	e-115	100
aC02016D05SK_c	**1-(5-phosphoribosyl)-5-[(5- phosphorib**	e-164	100
aCL1767Contig2	**Cytochrome P450 monooxygenase CY**	e-152	100
aCL271Contig1	**Beta xylosidase**	e-133	100
aCL7797Contig1	**Pol polyprotein**	e-118	100
(3x)aCL105Contig1	**Victorin binding protein**	3,00E-80	98
(3x) aCL3Contig25	**Lipid-transfer protein**	e-100	100
aCL172Contig2	**RD22-like protein**	1,00E-79	97
aCL5Contig7	**T20M3.14 protein**	9,00E-87	100
aCL5500Contig1	**Cytosolic ascorbate peroxidase**	3,00E-82	100
(6x)aCL6Contig16	**COR15**	1,00E-41	100

Contig	**Blast X**	**expect**	**Id**
aCL3072Contig1	Homogentisate geranylgeranyl transferase	9.00E-44	90
aCL3Contig25	Lipid-transfer protein	7.00E-95	100
aCL9Contig21	Late embryogenesis abundant protein Lea5	2.00E-74	95
aCL2182Contig1	SBT1 protein	e-155	99
aCL42Contig1	Expressed protein	e-132	94
aCL4812Contig1	T9A4.6 protein	e-113	100
aCL2371Contig2	OSJNBa0084A10.18	e-101	100
aCL1691Contig1	Pectin methylesterase precursor	e-120	98
aCL747Contig1	Thaumatin-like protein 1 precursor	e-134	99
aCL3488Contig1	ACC oxidase ACCO2	9.00E-47	98
aC18016A02Rv_c	Cytochrome P450 monooxygenase CYP83	e-127	100
aCL43Contig3	Ribulose bisphosphate carboxylase small cha	e-100	100
aCL1309Contig1	Putative GTPase	8.00E-58	98
aC08016H09SK_c	UPI00005491F6	e-165	100
aCL505Contig2	Glutamate decarboxylase 4a	4.00E-81	100
aCL203Contig1	Serine/threonine-protein phosphatase PP1 is	e-133	100
aCL5Contig4	Cytochrome P450 like_TBP	e-127	98
aCL6Contig12	COR15	1.00E-43	82
aCL859Contig1	Ferritin-3	1.00E-58	98
aCL691Contig1	Uridylate kinase	7.00E-59	90
aCL669Contig1	Putative phi-1 protein	1.00E-73	100

And more……

Gene dosage in the 39B3 mutation

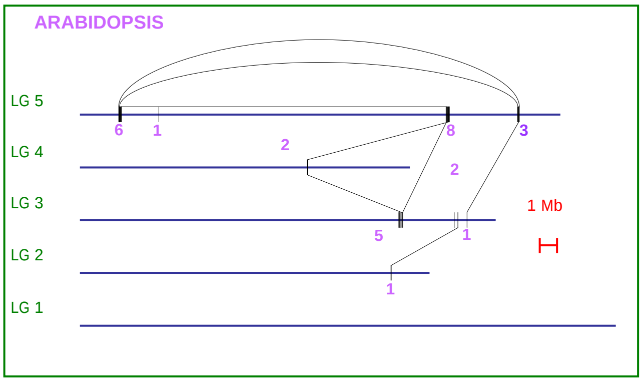
EST	GenBank ID	Unigene	aCGH		Real Time PCR	
			Mutant/wt ratio	P value	Gene dosage 39B3	Gene dosage 39E7
C05075A04	CX295702	aCL4690Contig1	0.59	0.10	0.56 ± 0.02	0.99 ± 0.09
IC0AAA9AA03	DY300024	aCL1915Contig2	0.62	0.12	0.60 ± 0.08	0.96 ± 0.04
IC0AAA16BA06	DY265056	aCL3317Contig1	0.62	0.10	0.60 ± 0.03	0.98 ± 0.03
C20009H03	CX308429	aC20009H03SK_c	0.63	0.10	0.50 ± 0.02	0.98 ± 0.11
C02007D11	CX288964	aCL766Contig1	0.65	0.13	0.56 ± 0.06	0.96 ± 0.11
C31005G06	?	aCL7097Contig1	0.65	0.12	0.59 ± 0.04	1.15 ± 0.07

Gene dosage in the 39E7 mutation

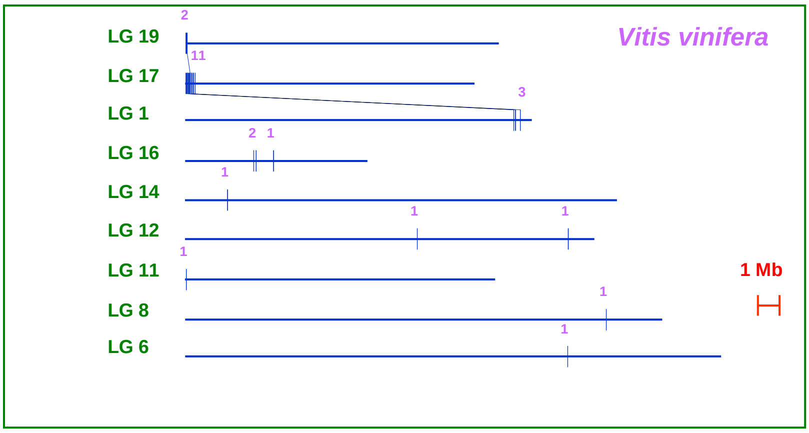
EST	GenBank ID	Unigene	aCGH		Real Time PCR	
			Mutant/wt ratio	P value	Gene dosage 39B3	Gene dosage 39E7
IC0AAA99DG07	DY300722	aCL5432Contig2	0.59	0.05	1.05 ± 0.13	0.59 ± 0.11
IC0AAA17BD05	DY265447	aCL6684Contig1	0.60	0.05	1.04 ± 0.12	0.59 ± 0.12
C34003D05	?	aCL6641Contig1	0.62	0.05	1.14 ± 0.19	0.58 ± 0.08
IC0AAA22BF01	DY267778	aCL3902Contig1	0.62	0.05	1.12 ± 0.10	0.64 ± 0.04
C05139C12	CX296347	aC05139C12SK_c	0.66	0.05	1.24 ± 0.22	0.61 ± 0.05
C01019E12	CX288357	aC01019E12SK_c	0.66	0.05	1.04 ± 0.07	0.63 ± 0.11

Are these putative deleted genes clustered together forming deletions?

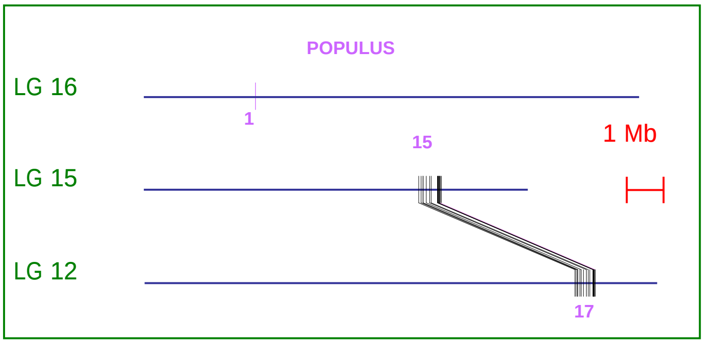
Mapping of plant homologues of the 39B3 mutation in sequenced genomes



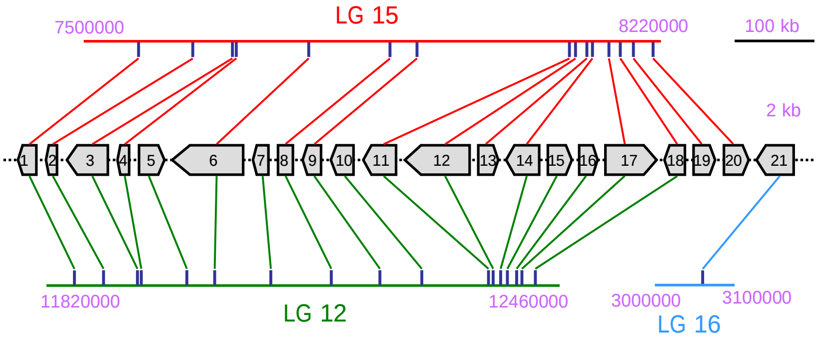
Mapping of plant homologues of the 39B3 mutation in sequenced genomes



Mapping of plant homologues of the 39B3 mutation in sequenced genomes



Clustering of homologues of the 39B3 deleted genes in the poplar genome



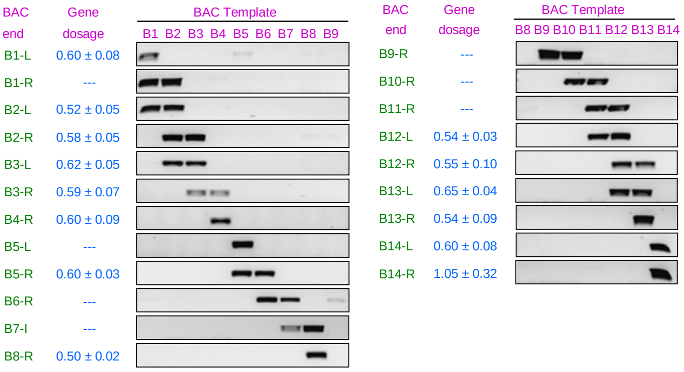
Are they clustered in this order also in citrus?

Validation through the generation of a partial physical map of the 39B3 deletion

Citrus BACs containing the 39B3 deletion

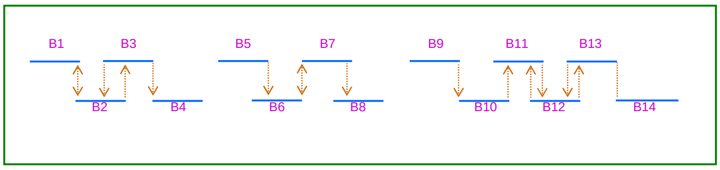
Nº	BAC	Ends	GenBank ID	BLASTX to plant proteins	E value
B1	CCL021E18	B1-L	ET070583	Nhf	--
		B1-R	ET070584	gi|91805627| hypothetical protein	9e-24
B2	CCL011O24	B2-L	ET086992	Nhf	--
		B2-R	ET086991	gi|7576215| hypothetical protein	7e-47
B3	CCER1037B12	B3-L	ET077105	gi|7576215| hypothetical protein	3e-90
		B3-R	ET077106	Nhf	--
B4	CCER1032N17	B4-L	ET101817	gi|25411577| probable retroelement pol polyprotein	2e-06
		B4-R	ET101816	Nhf	--
B5	CCL011N15	B5-L	ET087145	gi|6469119| mitochondrial phosphate transporter	5e-56
		B5-R	ET087144	Nhf	--
B6	CCER1045A09	B6-L	ET077286	gi|92895029| Polynucleotidyl transferase (retrotransposon protein)	8e-63
		B6-R	ET077285	gi|30027167| auxin response factor-like protein	6e-85
B7	CCH3037D01	B7-L	ET112059	gi|87240692| Helix-loop-helix DNA-binding	1e-21
B8	CCER1005N09	B8-L	ET079746	Nhf	--
		B8-R	ET079745	gi|79331867| AML1; RNA binding / nucleic acid binding	2e-09
B9	CCH3005L04	B9-L	ET081228	gi|33113977| putative copia-type pol polyprotein	2e-85
		B9-R	ET081227	gi|51968598| peroxisomal Ca-dependent solute carrier-like protein	2e-21
B10	CCER1033B14	B10-L	ET102435	gi|51968598| peroxisomal Ca-dependent solute carrier-like protein	2e-37
		B10-R	ET102434	Nhf	--
B11	CCL011K21	B11-L	ET086761	gi|25402907| protein F5M15.26 (retrotransposon protein)	4e-78
		B11-R	ET086760	gi|14334878| putative ATP-dependent Clp protease ClpD	4e-57
B12	CCER1033L08	B12-L	ET102525	gi|3297823| putative protein	2e-66
		B12-R	ET102524	gi|15027963| unknown protein	3e-22
B13	CCER1019D04	B13-L	ET098996	Nhf	4e-35
		B13-R	ET098995	gi|6729532| putative protein	3e-28
B14	CCL032E17	B14-L	ET094320	gi|6729532| putative protein	9e-34
		B14-R	ET094321	Nhf	--

Partial physical map of the 39B3 deletion

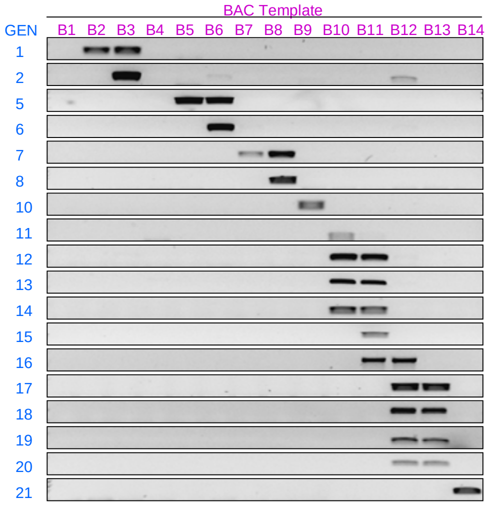


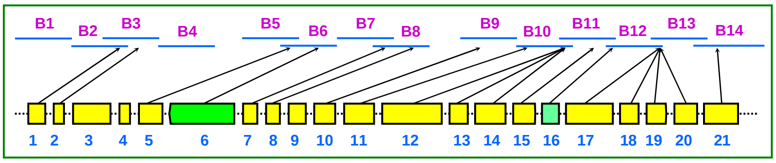
BAC end	Gene dosage
B1-L	0.60 ± 0.08
B1-R	---
B2-L	0.52 ± 0.05
B2-R	0.58 ± 0.05
B3-L	0.62 ± 0.05
B3-R	0.59 ± 0.07
B4-R	0.60 ± 0.09
B5-L	---
B5-R	0.60 ± 0.03
B6-R	---
B7-l	---
B8-R	0.50 ± 0.02

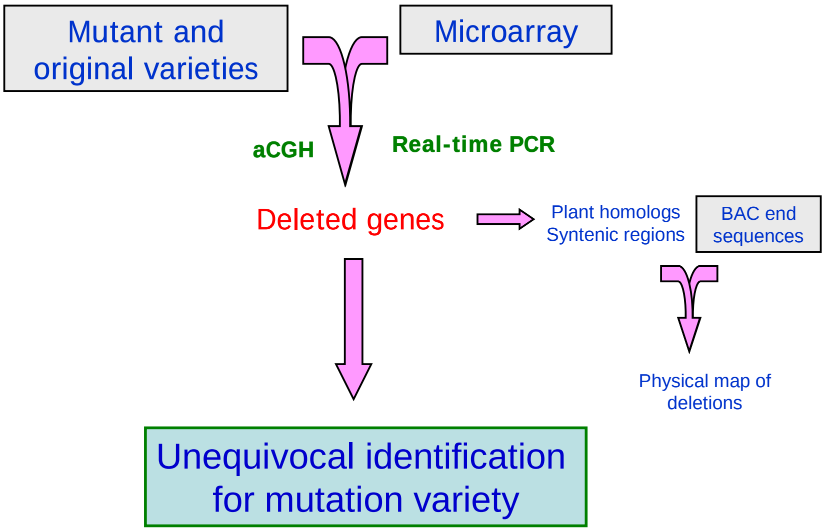
BAC end	Gene dosage
B9-R	---
B10-R	---
B11-R	---
B12-L	0.54 ± 0.03
B12-R	0.55 ± 0.10
B13-L	0.65 ± 0.04
B13-R	0.54 ± 0.09
B14-L	0.60 ± 0.08
B14-R	1.05 ± 0.32



Gene arrangement in the 39B3 deletion







Mutant and original varieties

Microarray

aCGH

Real-time PCR

Deleted genes

Plant homologs
Syntenic regions

BAC end sequences

Physical map of deletions

Unequivocal identification for mutation variety

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Lothar Willmitzer	MAX PLANCK (Alemania)
Luis Navarro	IVIA (Valencia)
M Isabel Minguez	CSIC (Sevilla)
Manuel Paneque	UNIVERSITY OF GLASGOW
Monserrat Robles	UPV (Valencia)
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