La trasformazione genetica nella vite: applicazioni, benefici e rischi

Prof. Bruno Mezzetti Ph.D.
Dipartimento di Scienze Agrarie, Alimentari ed Ambientali
Università Politecnica delle Marche
Email: b.mezzetti@univpm.it

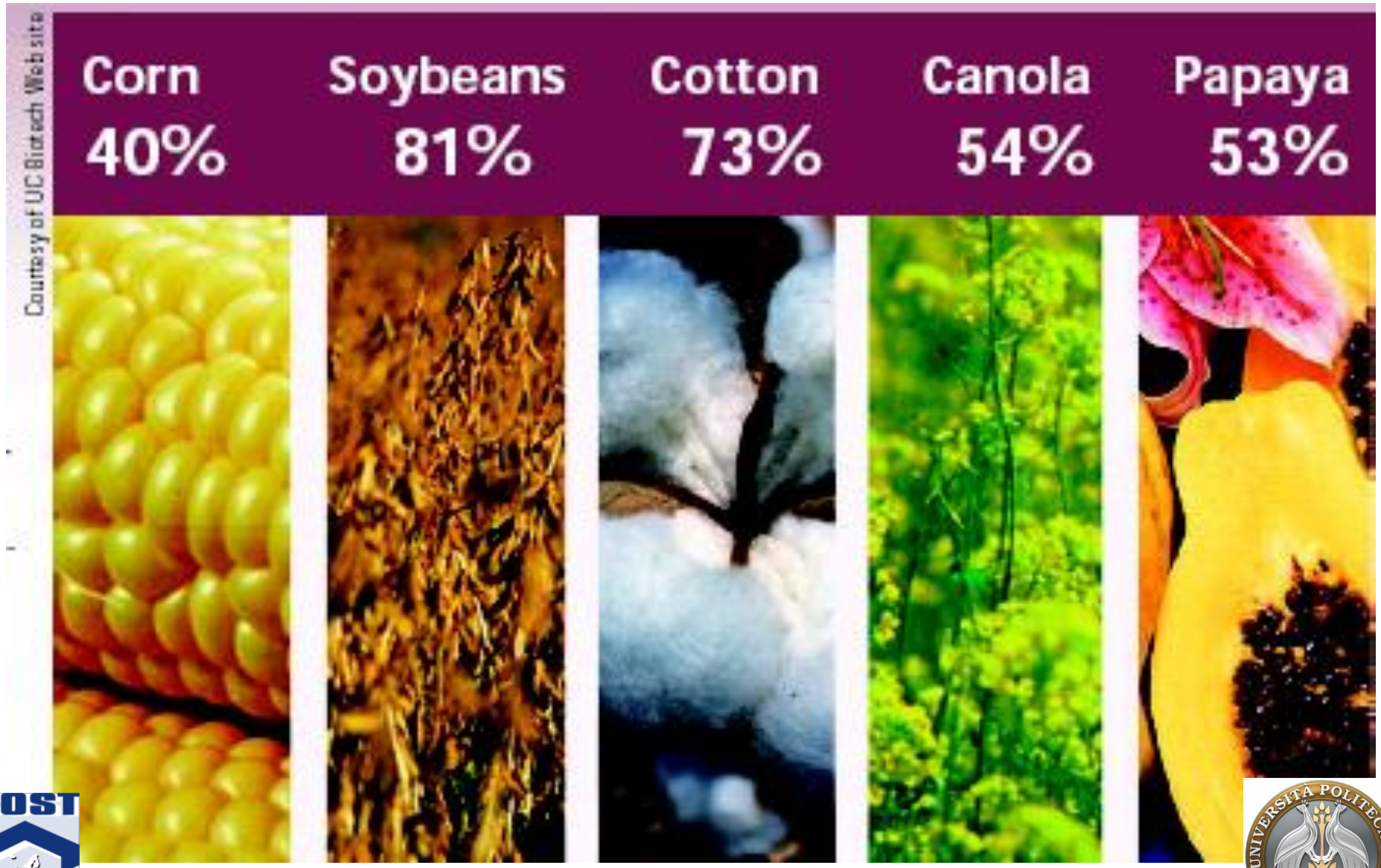
ACCADEMIA ITALIANA
DELLA VITE E DEL VINO

GLI O.G.M. IN VITICOLTURA

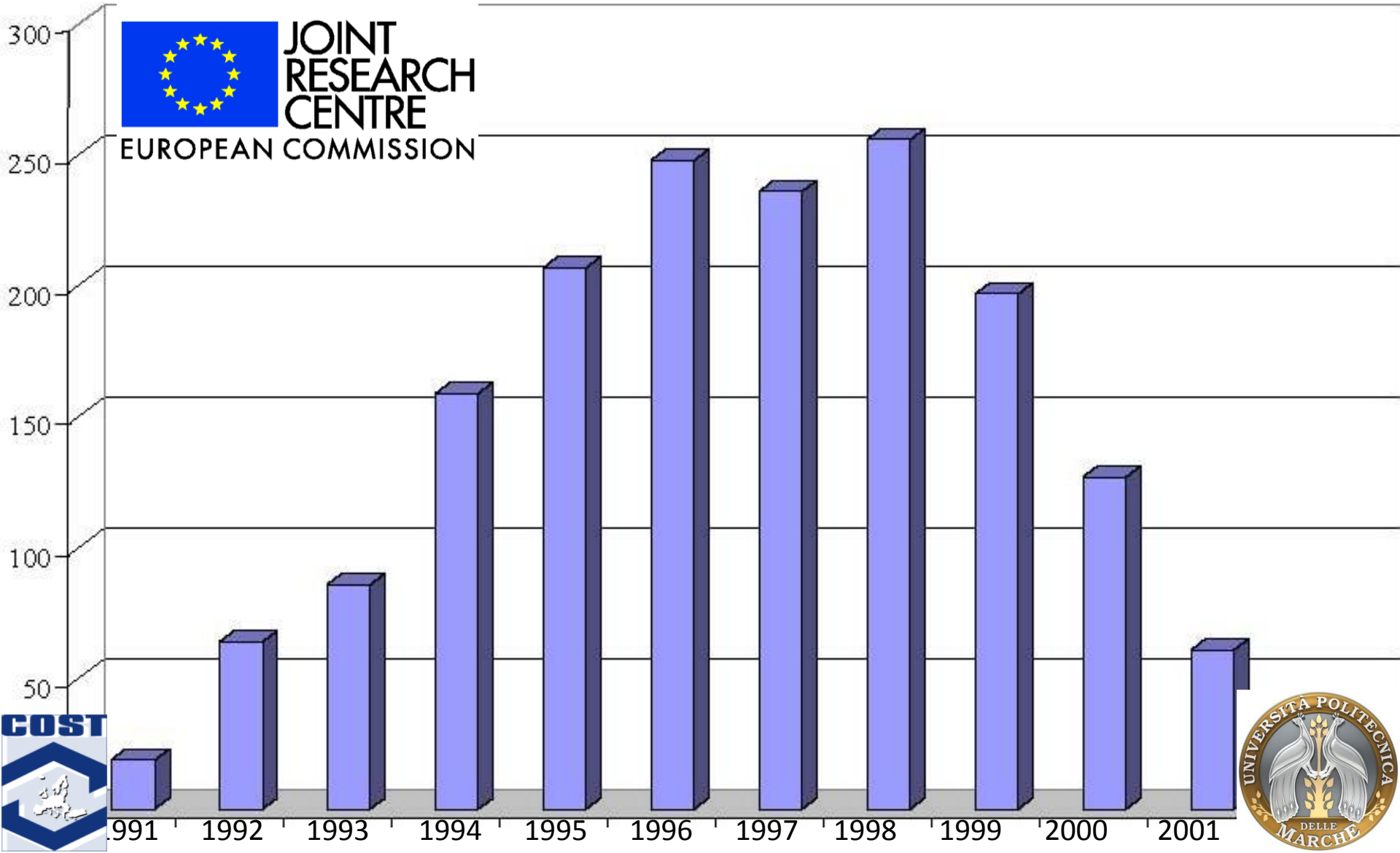
sabato 3 dicembre 2011
la Biblioteca Internazionale "LA VIGNA" di **Vicenza**

Parte 1ª

GM MOST CULTIVATED CROPS

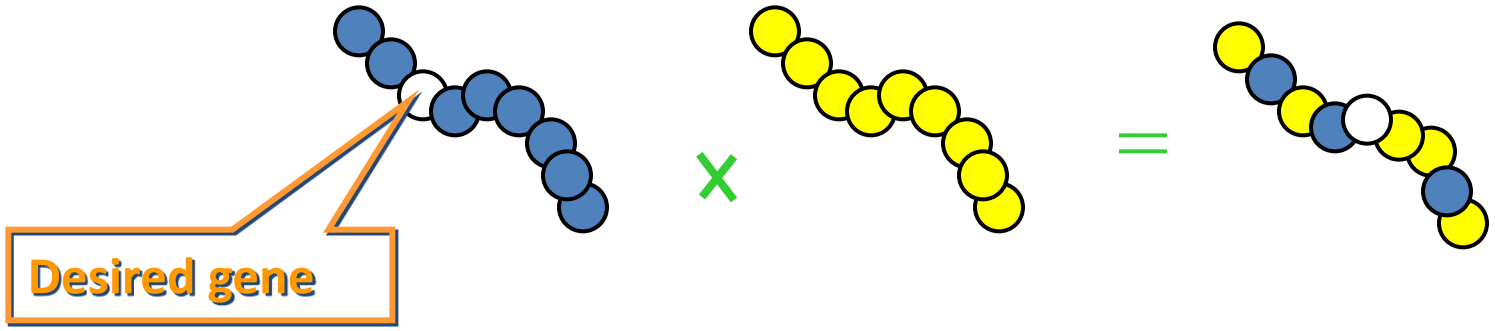


GM Research Trend in Europe 1991-2002
Total number of permits and notification approved per year

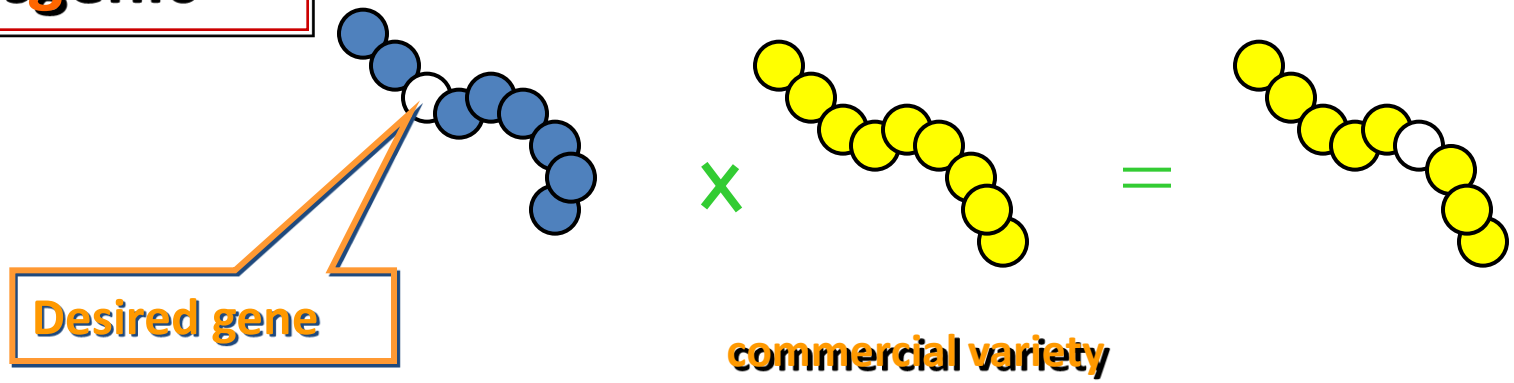


Traditional

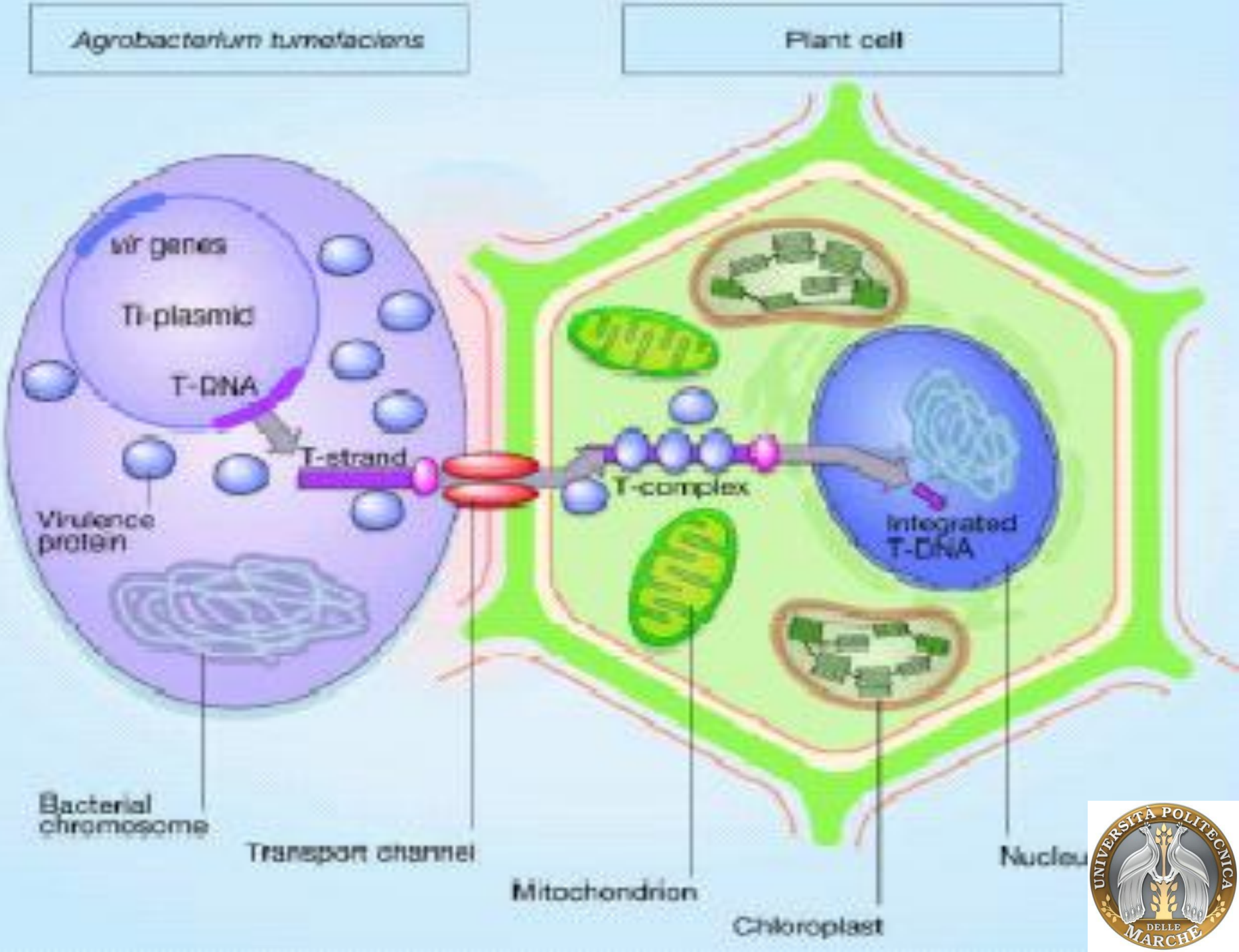
commercial variety



Transgenic



commercial variety

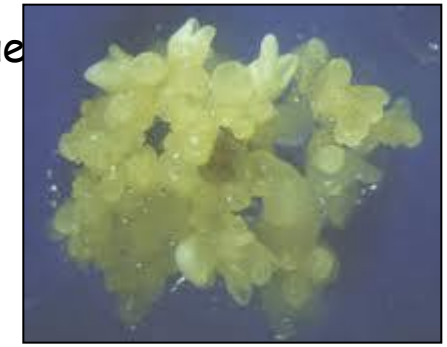

Agrobacterium tumefaciens
Plant cell
vir genes
Ti-plasmid
T-DNA
T-strand
Virulence protein
Bacterial chromosome
Transport channel
T-complex
Integrated T-DNA
Mitochondrion
Chloroplast
Nucleu

Genetic transformation of grape

via-Embryogenesis

transformation of embryogenic calli obtained from different tissue

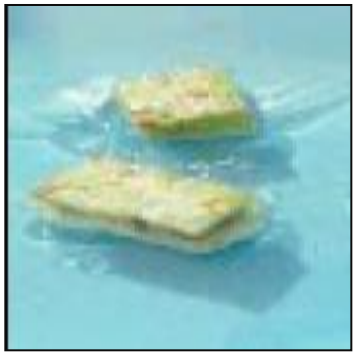
- zygotic embryos
- leaves
- ovaries
- anther filaments



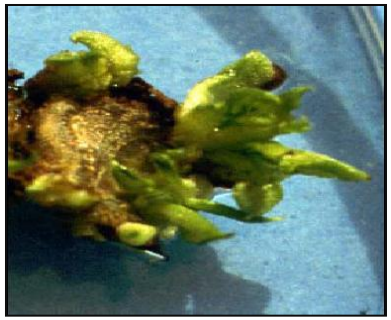
Via-Organogenesis

based on the formation of a meristematic bulk with a high regenerative capacity, using adventitious shoots as a starting material.

Meristematic Bulk from adventitius shoot



Meristematic tissues

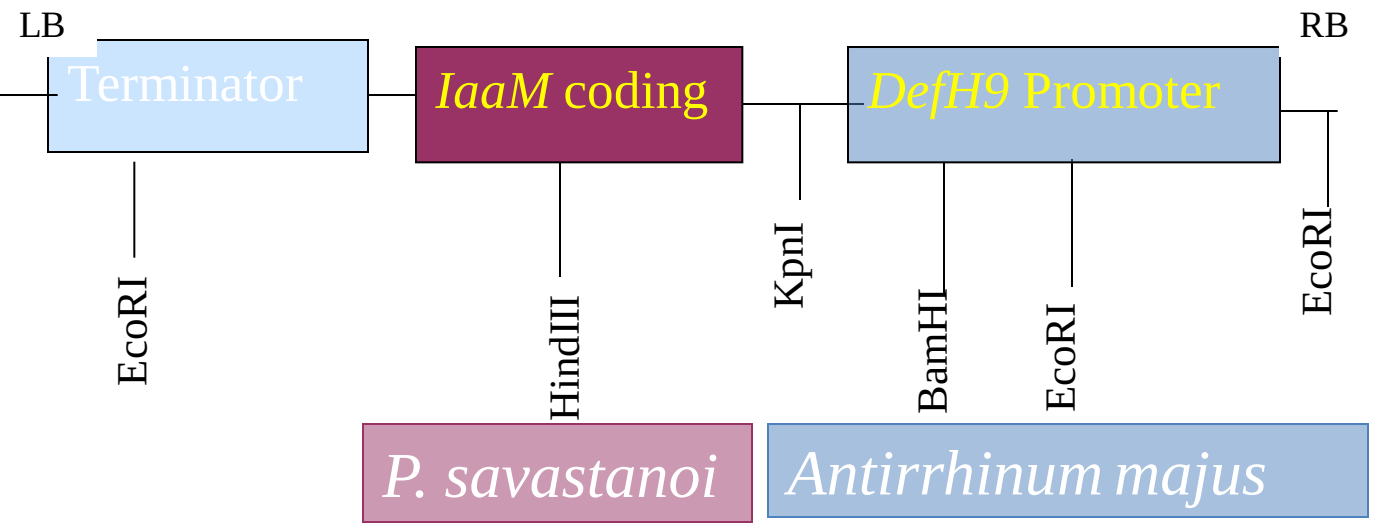


Regeneration

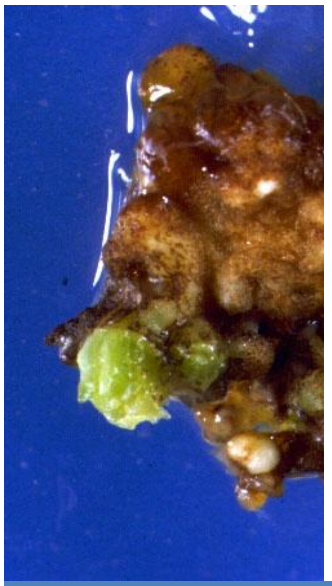
micropropagation

genetic transformation

DefH9-IaaM Gene (Spena – UniVR)

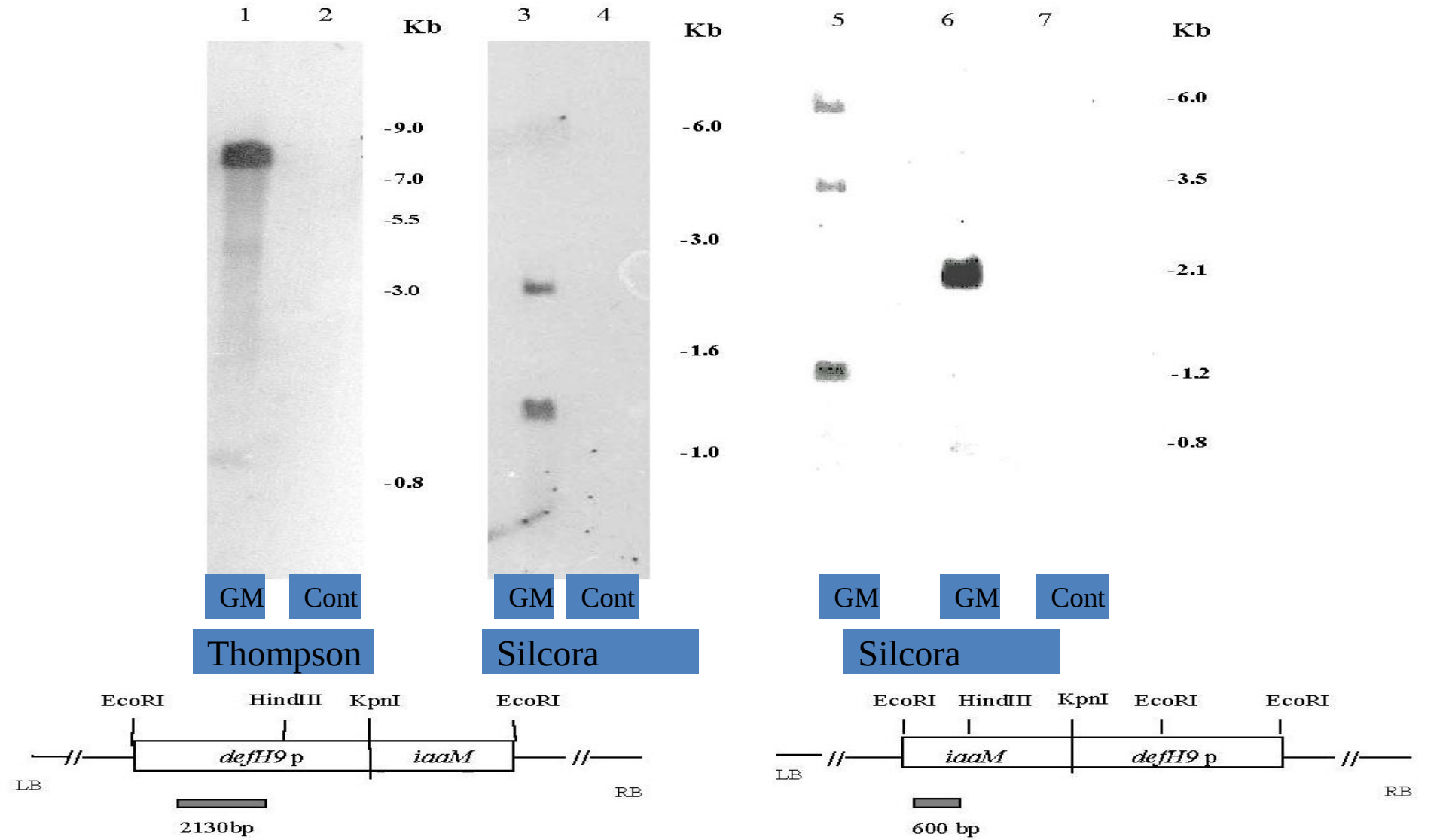


It confers expression within the placenta and the ovules and it has been used to confer parthenocarpic fruit development to several plant species and varieties (Rotino et al., 1997)



From regeneration and selection to the open field (2001)

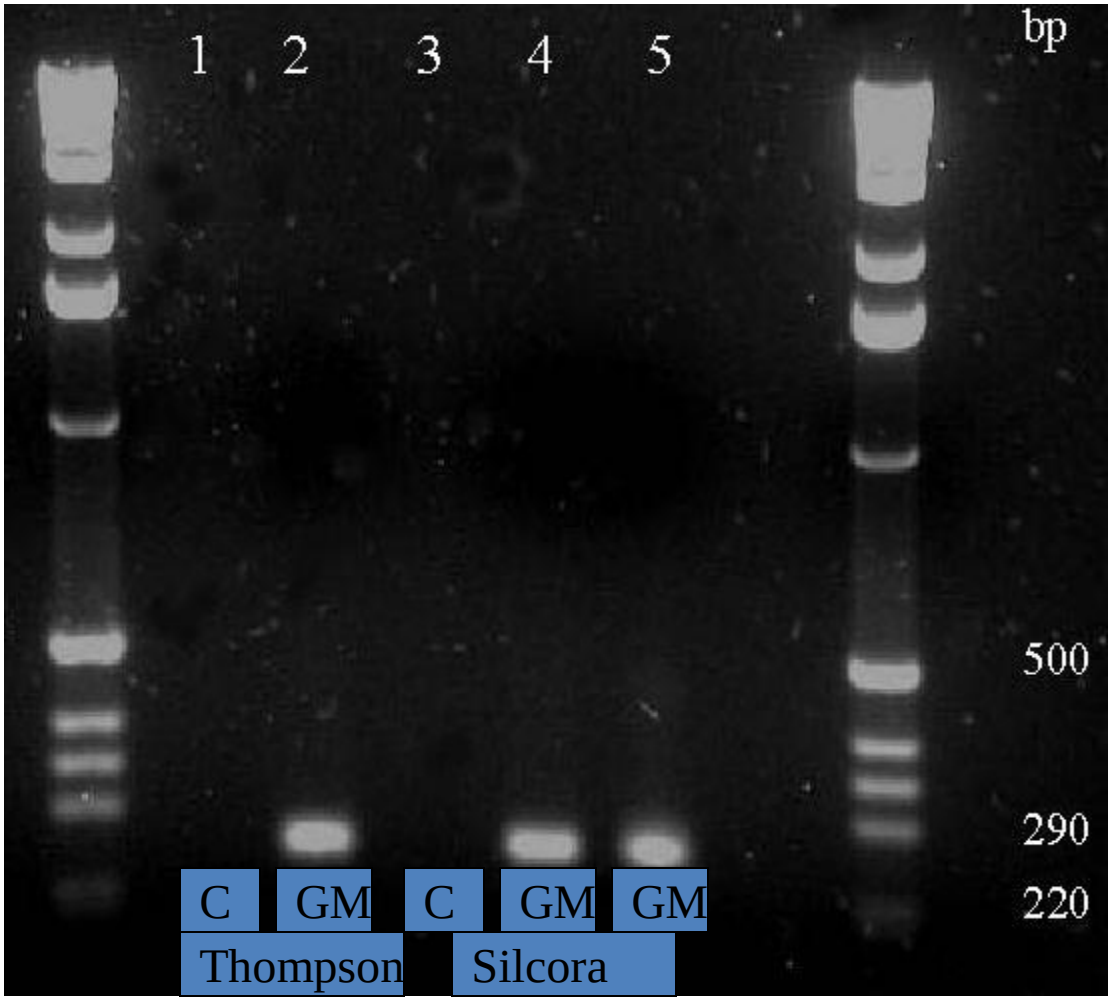
DefH9–iaaM Table Grape (Genomic DNA digested with *Hind*III)



Schematic drawings of the constructs used for transformation of Silcora (right) and Thompson Seedless (left) plants and probes indicated with grey boxes. Only restriction sites relevant for Southern analysis are indicated.

DefH9–iaaM expression in Table Grape

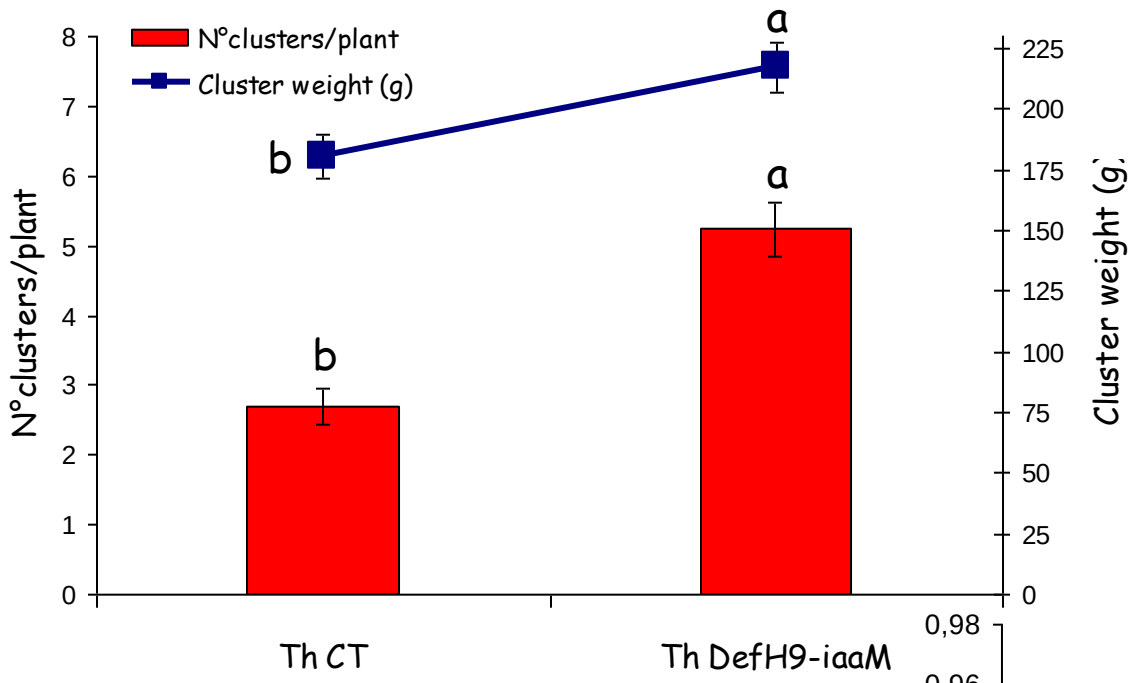
RT-PCR analysis performed with single strand cDNA synthesized from mRNA extracted from young flower initials of Thompson Seedless and Silcora control and transgenic plants.
The amplification product of 266 bp corresponds to the 5' end of the spliced *DefH9-iaaM* mRNA.



Flower initials transgenic for the *DefH9-iaaM* gene had an IAA content higher than controls (data not reported).
The *DefH9-iaaM* auxin-synthesizing gene does not however inhibit grape fruit ripening.

The experimental trial:
'Thompson Seedless' Control and GM line: 32 plants each
'Silcora' Control and GM lines (line A and line B): 16 plants each
Vines were spaced at 2.5 x 1.5 m, trained with the 'double guyot' system.

1. Thomphson seedless yield

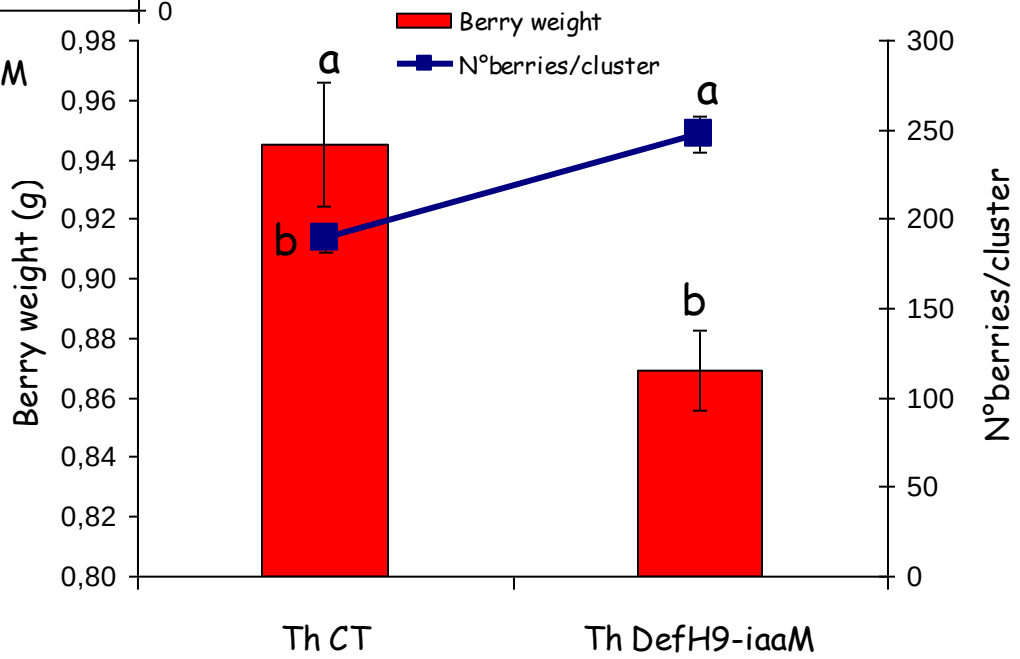


DefH9-iaaM gene effects on plant production, fruit and berry size cv. 'Thompson seedless'

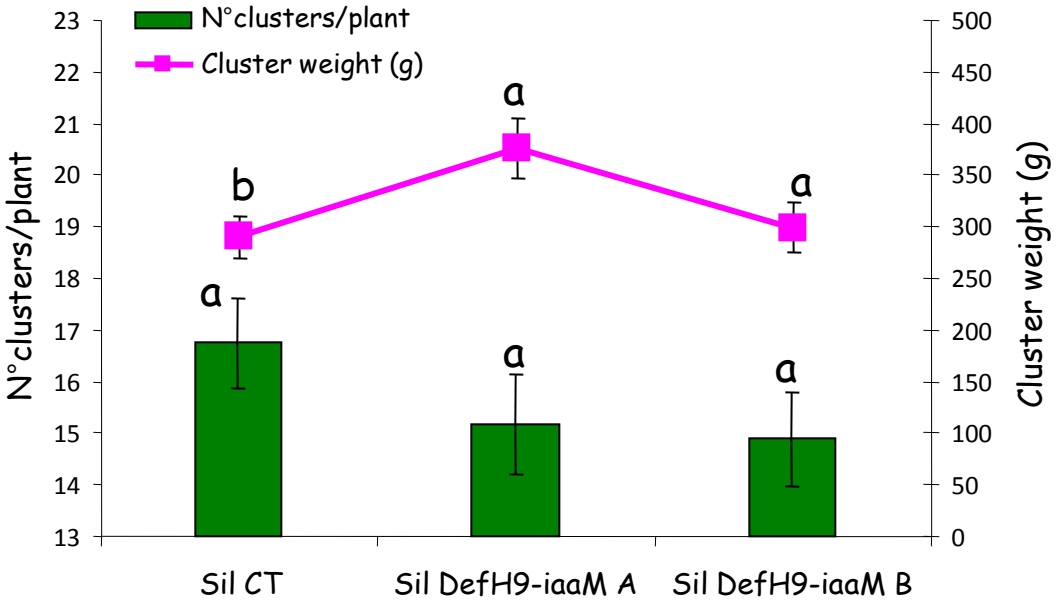
Production cycles 2004-05-06

Mean±SE

Duncan's statystical test, $P \leq 0.05$

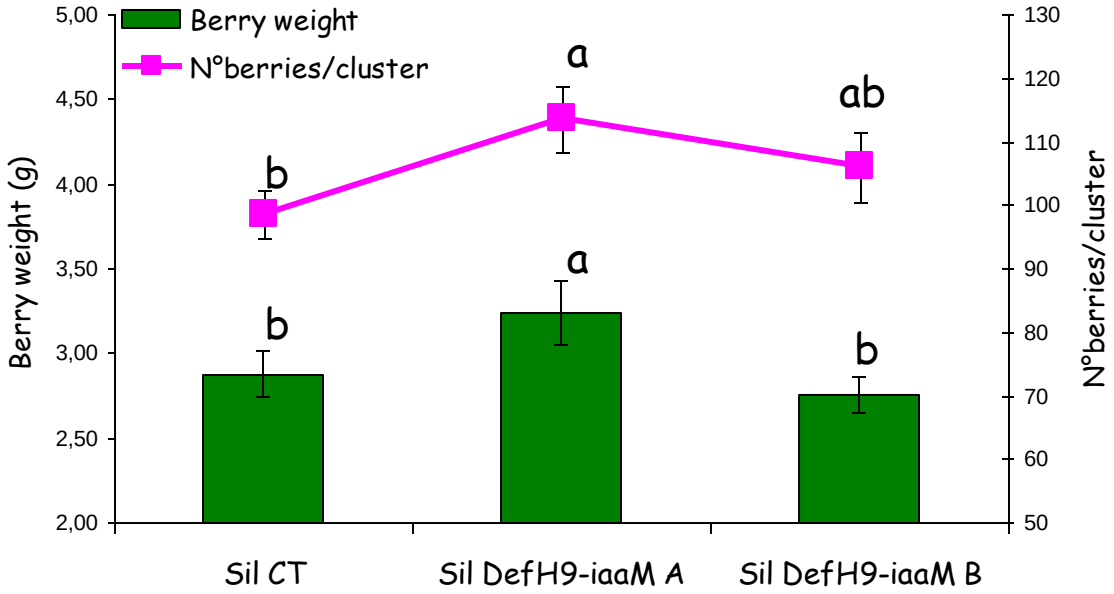


1. SILCORA Yield



DefH9-iaaM gene effects on plant production, fruit and berry size cv. 'Silcora'

Production cycles 2004-05-06
Mean±SE
Duncan's statystical test, P ≤ 0.05



DefH9-iaaM gene effects on berry quality and nutritional values

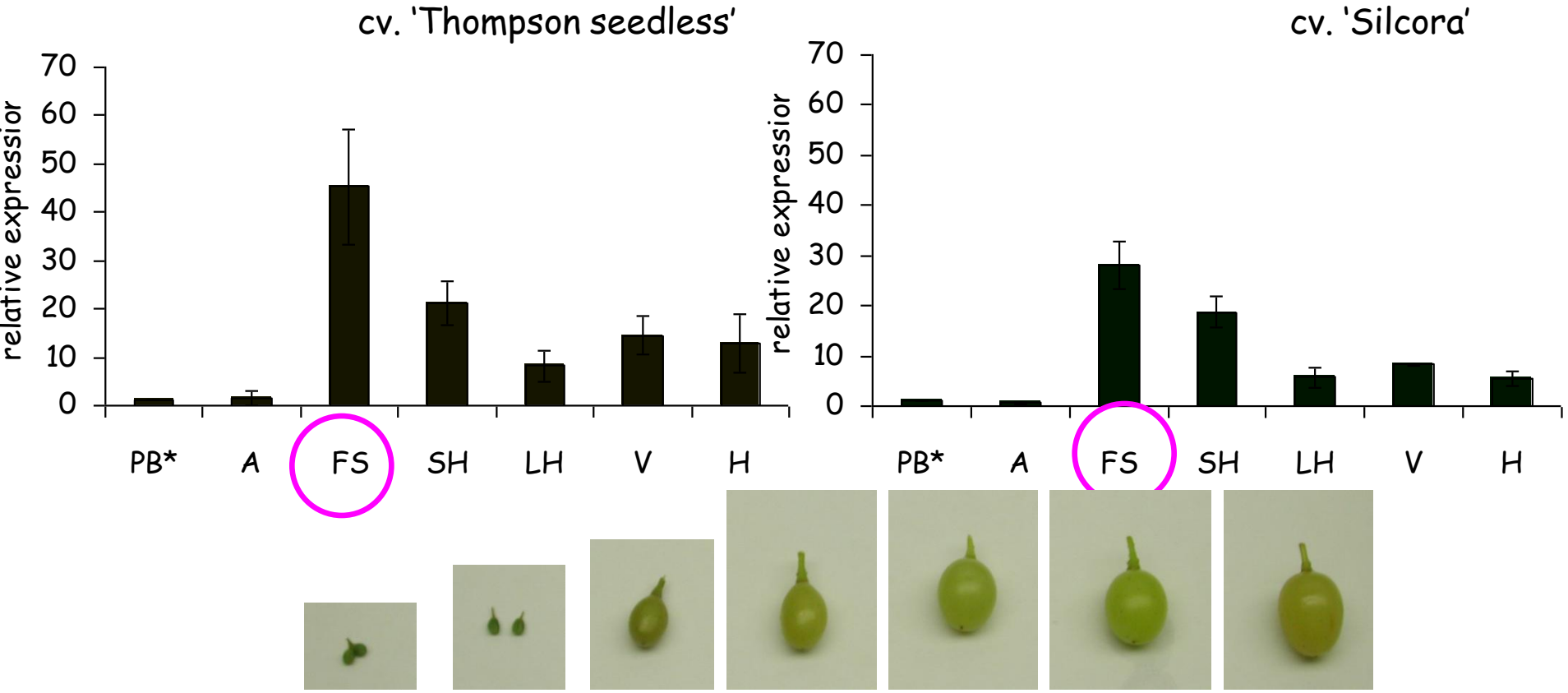
Produc. cycles 2004-05-06	Soluble Solid Content (°B)	Titratable Acidity	pH	Tartaric Acid(g/L)	MalicAci (g/L)	Citric Acid (g/L)
Th CT	18.83±0.35 ns	9.62±0.35 a	3.14±0.02 b	8.58±0.27 ns	3.36±0.23 a	0.30±0.02 a
Th GM line	18.7±0.38 ns	7.97±0.24 b	3.22±0.03 a	8.35±0.19 ns	2.42±0.32 b	0.22±0.01 b
Sil CT	15.19±0.60 ns	5.48±0.40 ns	3.25±0.05 ns	7.03±0.36 ns	1.28±0.18 ns	0.11±0.01 ns
Sil line A	15.62±0.39 ns	5.43±0.39 ns	3.30±0.04 ns	6.67±0.32 ns	1.47±0.20 ns	0.12±0.01 ns
Sil line B	14.81±0.51 ns	5.92±0.36 ns	3.23±0.05 ns	7.04±0.33 ns	1.27±0.14 ns	0.10±0.01 ns

Mean±SE;
Duncan's statystical test, P ≤ 0.05

Produc. cycles 2004-05-06	TPH Mg GA/gfrutto	TEAC TroloxE(umoli/g)
Th CT	1.02±0.03 b	3.34±0.10 a
Th GM *line*	1.13±0.04 a	3.08±0.7 b
Sil CT	1.57±0.09 b	5.98±0.30 b
Sil GM line A	2.10±0.07 a	7.54±0.28 a
Sil GM line B	1.93±0.13 a	7.13±0.42 a

Mean±SE;

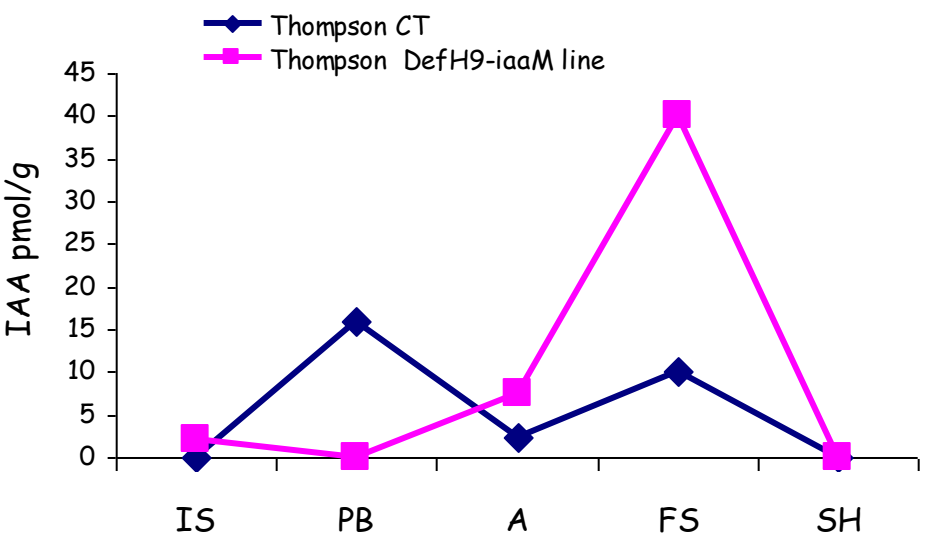
DefH9-iaaM gene expression during berry development

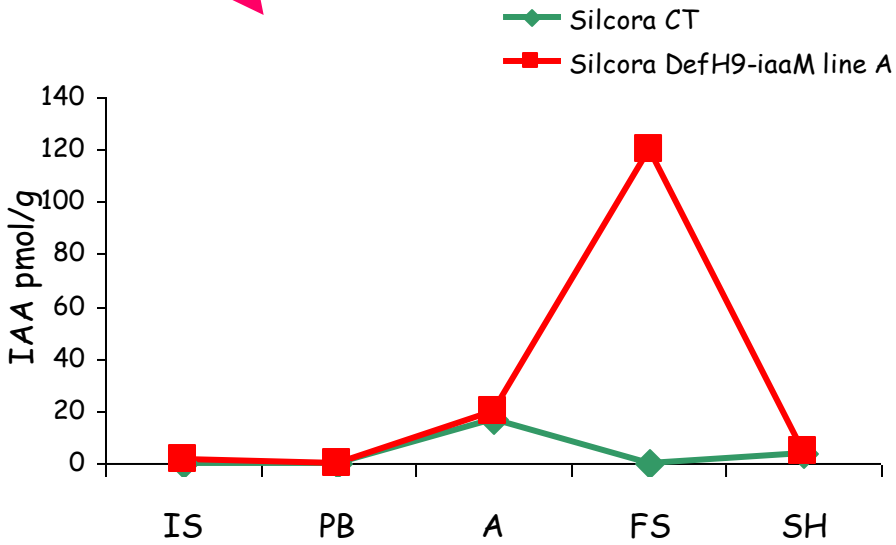


Fruit developmental phases: PB= pre-blooming; A= antesys; FS= fruit-set; SH= small hard fruit; LH= large hard fruit. Values are referred to PB, arbitrary set to 1.

IAA content during berry development

GC-MS analysis on tissues samples
Cv 'Thompson seedless' and 'Silcora' (control and GM line)





Fruit developmental phases: IS=inflorescences separated; PB=pre-blooming; A= antesys; FS=fruit-set; SH=small hard fruit

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Parte 2ª

NOW RUNNING PROJECTS:

Application of post transcriptional gene silencing (PTGS) to pathogen resistance in grape and functional genomics

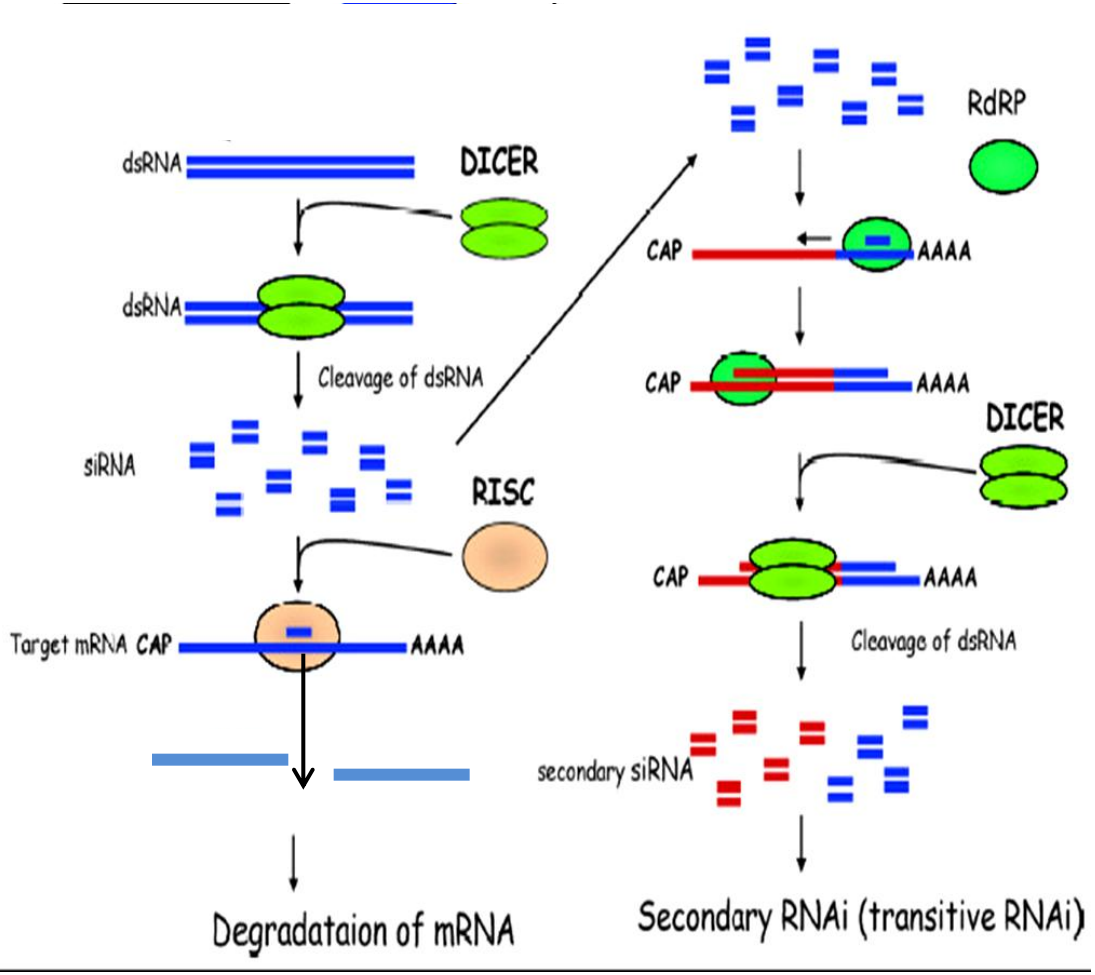
I. Study of techniques to confer virus resistance in grapevine through PTGS.

II. To identify grapevine homologous of the AUCSIA tomato genes, study their expression and investigate their function through PTGS-mediated genetic suppression.

Implementation of a grape transformation method based on organogenesis

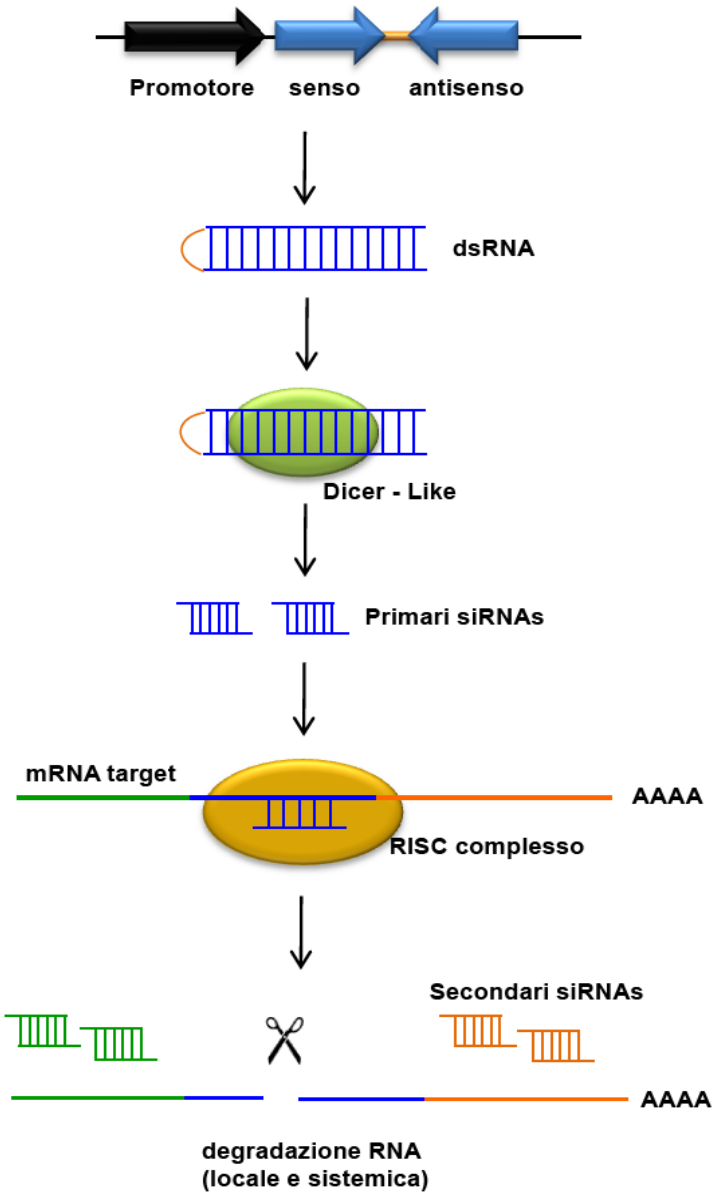
Post transcriptional gene silencing (PTGS)

Post-trascriptional gene silencing (PTGS) is an ubiquitary mechanism of adaptative defence against viruses and mobile genetic elements



Double stranded RNA can be produced by inverted repeats, viral RNA or by the action of RNA-dependent RNA polymerase

PTGS mechanism has proved to be a useful biotechnological tool to produce plants resistant to viruses.



Hairpin constructs, carrying parts of viral genome can be introduced in plants leading to the inhibition of viruses replication

I. Study of techniques to confer virus resistance in grapevine through PTGS

Fanleaf disease

➢ The principal causative agent of this disease is the *Grapevine fanleaf virus* (GFLV), often associated with other viruses such as the *Arabis Mosaic Virus* (ArMV).

➢ Transmission:

- medium and long distance by propagation of infected material
- short distance by nematodes (*Xiphinema index* for *GFLV* and *Xiphinema diversicaudatum* for *ArMV*)

➢ The symptoms include:

- dwarfism
- reduced fruit productivity and quality
- alteration of leaf morphology

Leafroll disease

➢ The disease is ascribed to different viruses belonging to Closterovirus and Ampelovirus genera

➢ 5 GLRaV (*Grapevine LeafRoll – Associated Virus*): GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5 has been identified

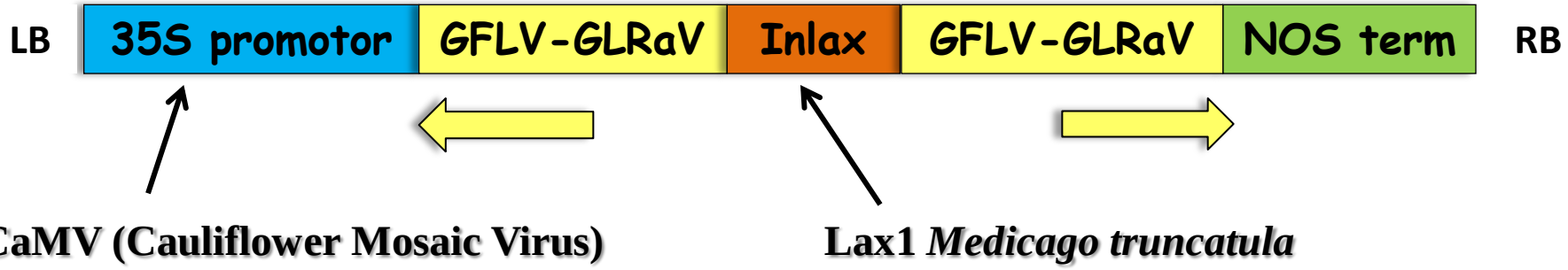
➢ Transmission:

- infected material
- insects (*Planococcus ,Pseudococcus and Pulvinaria vitis*)

THE hpCONSTRUCT

Schematic drawing of the construct

hpViruses GFLV-GLRaV



The hairpin construct contains:

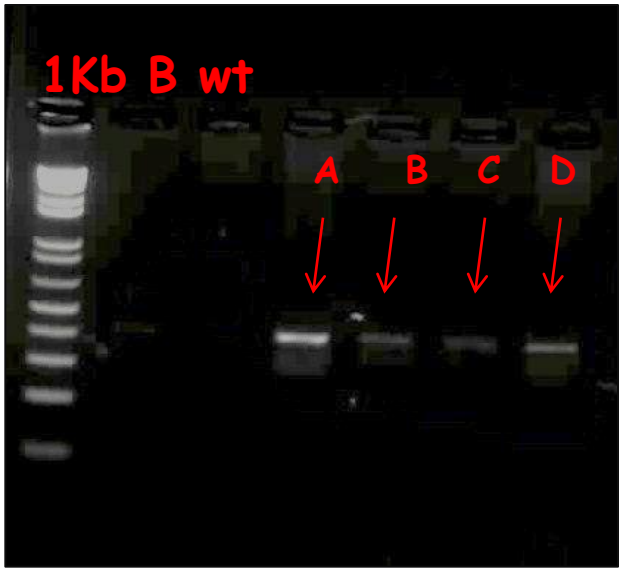
- a sequence of 200 bp of the GFLV RNA-dependent RNA polymerase gene
- a sequence of 202 bp of the GLRaV3 RNA-dependent RNA polymerase gene of the italian GLRaV3 isolate

Nicotiana benthamiana is a model plant for viral infection studies

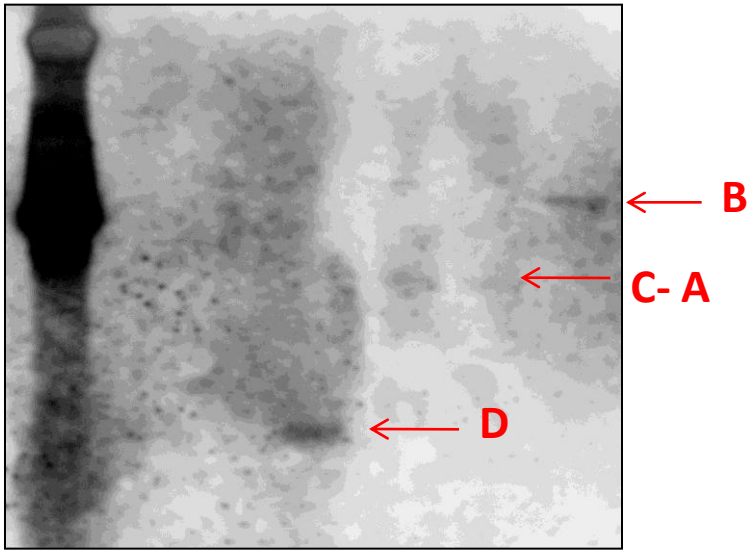
Genetic transformation with hpViruses GFLV-GLRaV

Nicotiana benthamiana independent lines transformed with the hpViruses GFLV-GLRaV construct

PCR analysis



Southern blot analysis



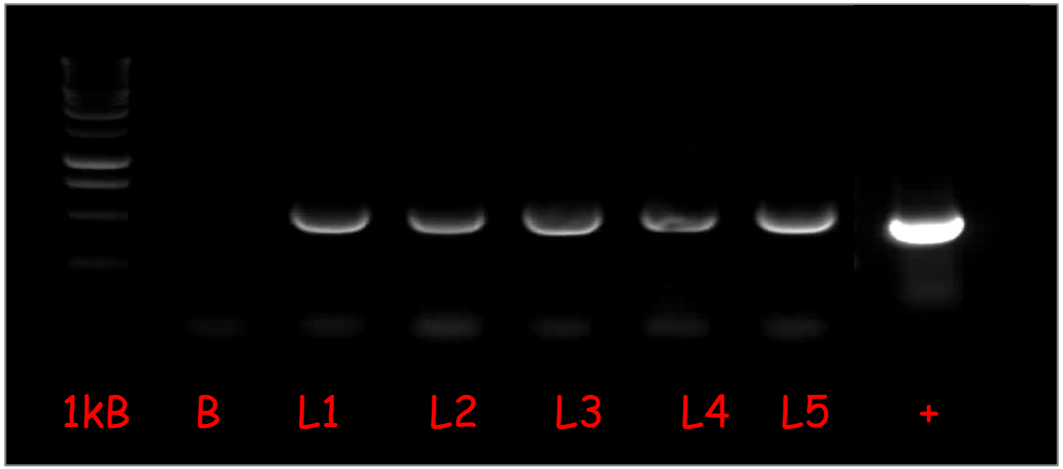
The T0 plants were backcrossed with wild-type plants and the T1 progeny were selected on kanamycin

The transgenicT1 progeny plants were mechanically inoculated with the ArMV

Genetic construct containing only regulatory regions has been used as control in the infection experiments

CaMV (Cauliflower Mosaic Virus)

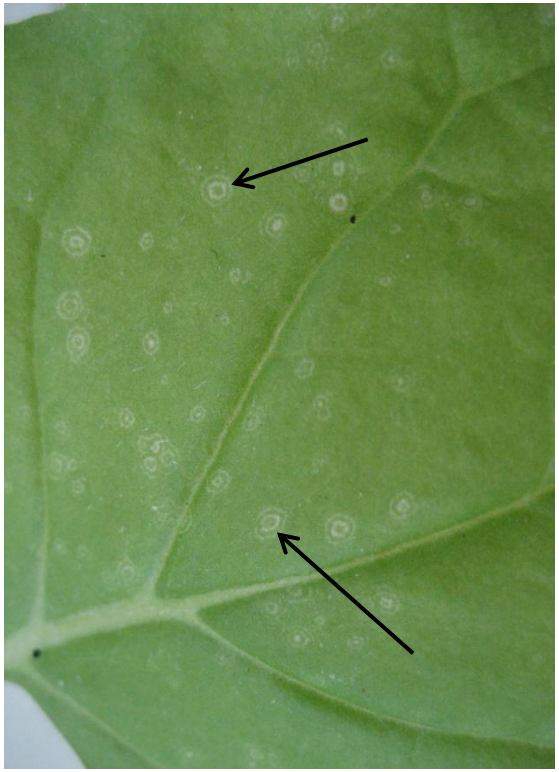
Lax1 *Medicago truncatula*



Five lines positive by PCR analysis

Inoculation of Nicotiana benthamiana with ArMV

In collaboration with Prof.ssa Annalisa Polverari



Local symptoms:

- chlorotic mottling

Sistemic symptoms:

- chlorotic bands along the veins
- curling of the leaves

To evaluate the systemic distribution of the ArMV virus 2-3 apical leaves were sampled two weeks after inoculation for the ELISA assays.

Sample	Absorbance 450 nm
B	**0.098**
Control +	**1.873**
hpViruses GGLV-GLRaV B5	2.030
B6	**0.095**
C6	1.911
C7	2.055
C8	**0.096**
D1	1.618
D3	2.036
D4	**0.099**
D6	1.893
Prom/intr/term F1	1.873
F5	1.795
G2	1.652
G5	1.870
G8	**0.098**
G10	1.864
G11	1.778
S1 (no inoculated)	0.104
S2 (no inoculated)	0.097
C.quinoa	2.060

Preliminary data seem to indicate a greater tendency in plants transformed with the construct hpViruses GFLV-GLRaV to limit the spread of the virus compared to wild-type plants.

GENETIC TRANSFORMATION AND RIGENERATION of GRAPEVINE (V.vinifera L.) with the *hpViruses GFLV-GLRaV* CONSTRUCT *and the 35S-GFP CONSTRUCT*

Proliferating shoots were subjected to chemical and mechanical treatments to induce the formation of meristematic bulks characterized by a strong capacity to differentiate adventitious shoots.

- **Initiation and maintenance of meristematic bulk**

CORVINA

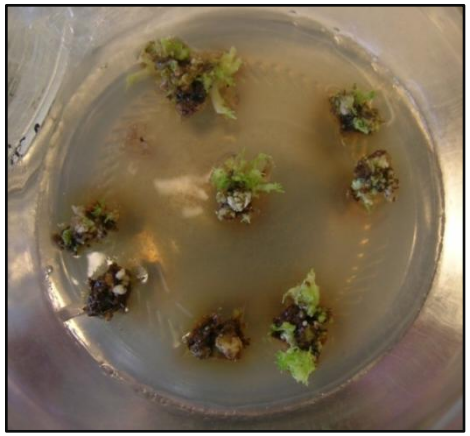
PINOT

1103 Paulsen

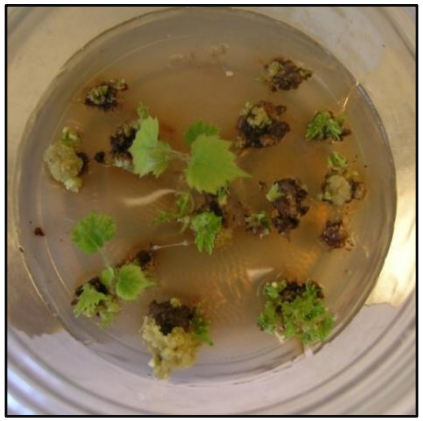
- **Agrobacterium tumefaciens infection**

Slices (1cm^2,2 mm) obtained from the meristematic bulk were dipped in the bacterial suspension (*A.tumefaciens* strain GV2260 harbouring construct hpVirusesGFLV-GLRaV and *A.tumefaciens* strain GV2260 harbouring construct GFP.)

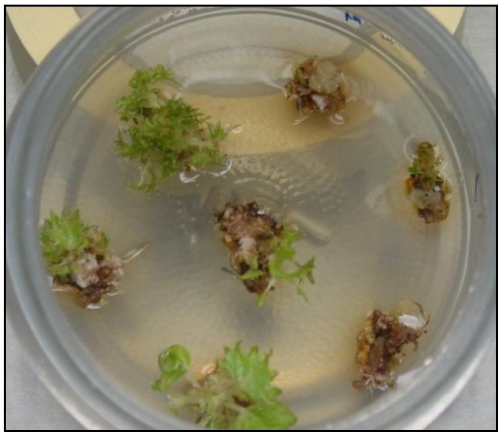
• Shoot regeneration



CORVINA



PINOT



1103 PAULSEN

The regeneration tests done on the cultivar Corvina evidenced a low regenerative ability as compared to Pinot noir and 1103 Paulsen

• Selection and rooting

I. Prolonged selection on medium containing kanamycin 25mg l^{-1}

Rooting of kanamycin 50mg l^{-1}

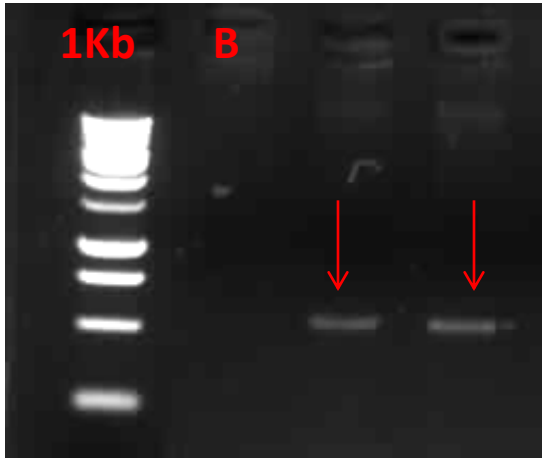
II. Selection at increasing concentrations of kanamycin (25mg l^{-1}, 35 mg l^{-1} and 50 mg l^{-1})

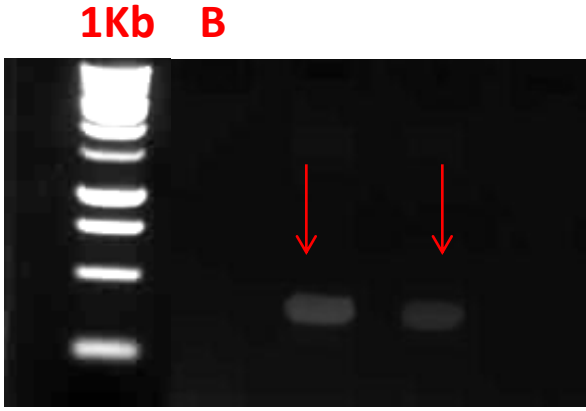
Rooting of kanamycin 35-50mg l^{-1}

I. Selection of kanamycin 25mg l^{-1} and rooting of kanamycin 50mg l^{-1}

After nine-ten months of selection the shoots obtained were transferred on rooting substrate

Two rooted CORVINA lines positive by PCR analysis





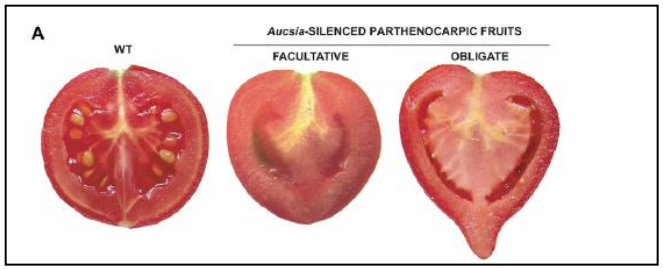
Pinot noir and 1103Paulsen transformed with the hpVirusesGFLV-GLRaV construct

II. Selection at increasing concentrations of kanamycin
(25mg l^{-1},35 mg $l^{-1}$and 50 mg l^{-1})

Rooting of kanamycin 35-50mg l^{-1}

Aucsia silenced plants

- Partenocarpic fruit



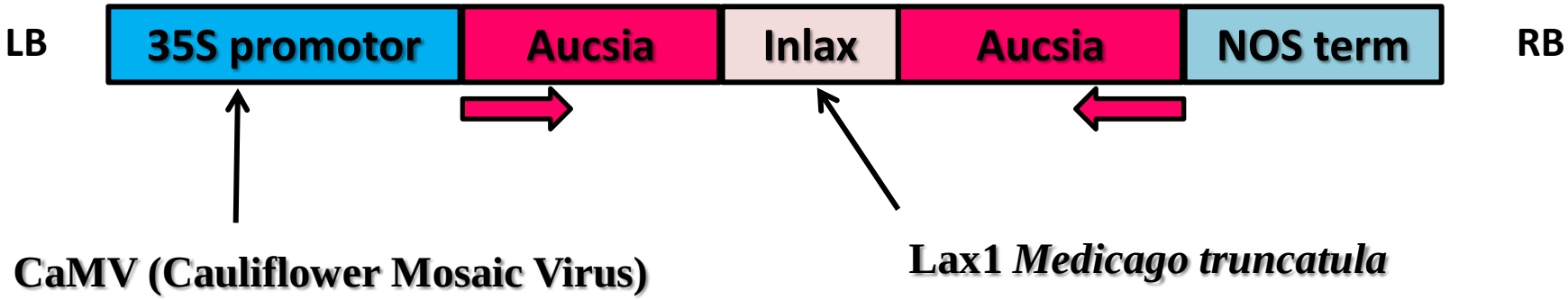
- Increase in IAA content of pre-anthesis in flower buds

Lines	Total IAA content (nmol g^{-1})
L276 1-1	113.6 ± 0.99
L276 7-1	261.0 ± 1.41
L276 WT	1.9 ± 0.33

- The fruits are reduced in size and weight

Lines	Fruit Set		Average weight (g)	
	n° of fruits/ n° of emasculated flowers	%	Emasculated	Selfed
L276 #1-1	4/9	44	13.49 ± 0.85***	38.98 ± 2.70***
L276 #4-1§	7/7	100	35.47 ± 12.03**	38.43 ± 21.10*
L276 #7-1	6/11	54	28.77 ± 4.25***	34.29 ± 2.04***
L276 #8-2	10/15	67	40.14 ± 2.93***	48.38 ± 3.13*
L276 WT	0/15	0	-	81.29 ± 4.39
INB777 #7-1	20/25	80	18.27 ± 1.61***	48.72 ± 5.76**
INB777 #13-3	14/20	70	18.47 ± 1.36***	61.04 ± 5.55*
INB777 WT	0/20	0	-	77.40 ± 4.24

THE VvAucsia1hp CONSTRUCT



Schematic drawing of the construct used for *V. vinifera L* transformation.

The hairpin construct contains:

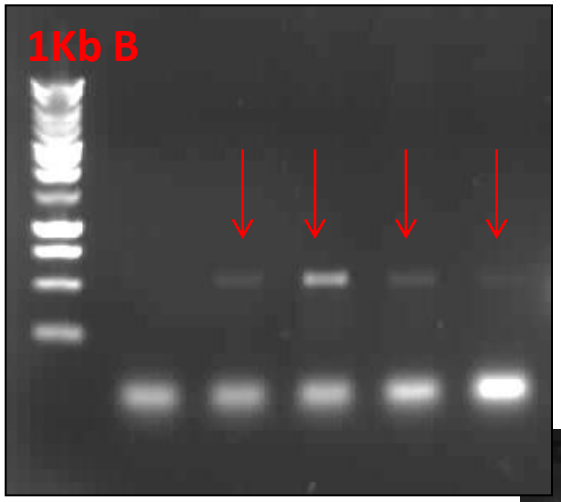
- a sequence of **200 bp** of the **VvAUCSIA1 gene**

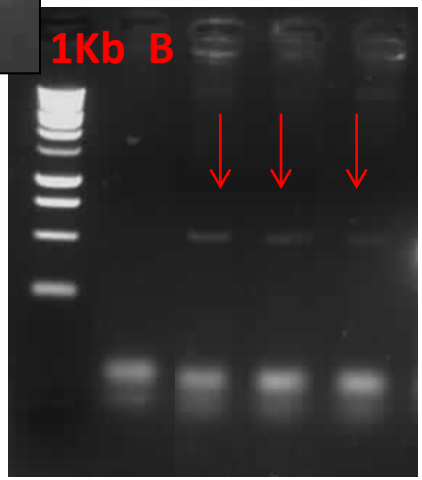
RESULTS

I. Selection of kanamycin 25mg l^{-1} and rooting of kanamycin 50mg l^{-1}

After nine months of selection the shoots obtained are transferred on rooting substrate

Seven lines rooted were positive to PCR analysis

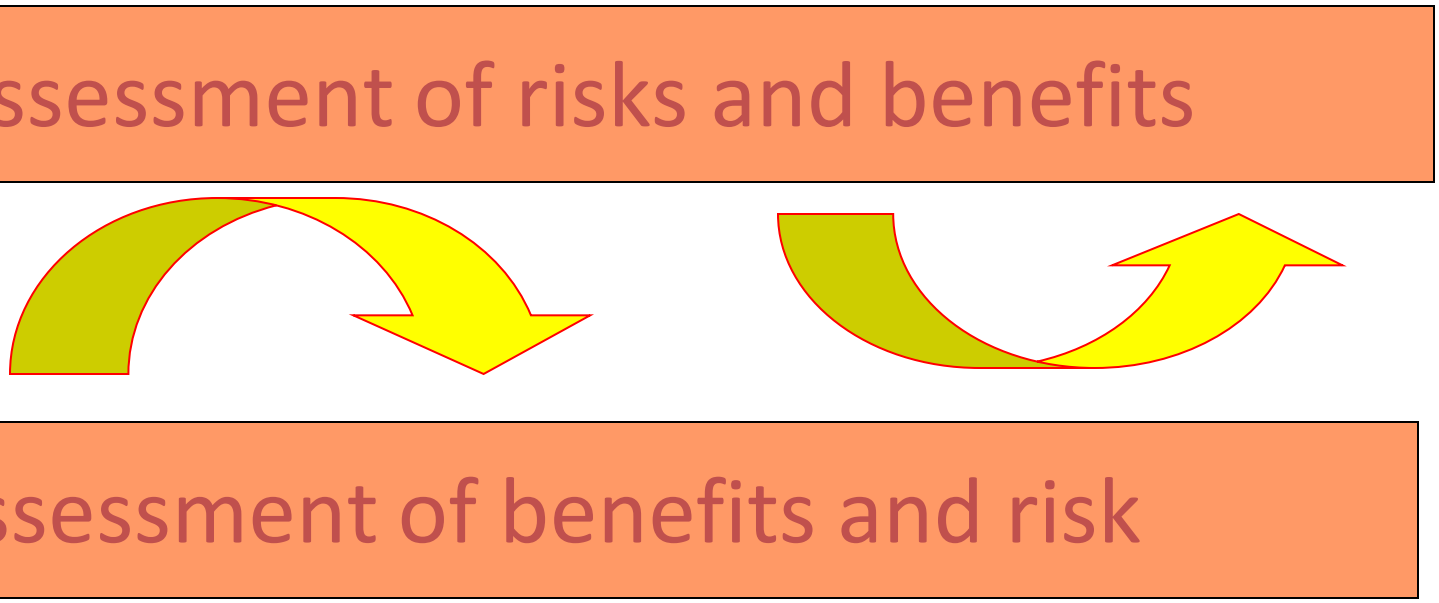




LA VITE TRANSGENICA NEL MONDO

- **Dennis J. Gray**
- **Professor of Developmental Biology**
- **University of Florida, IFAS**
  Mid-Florida Research & Education Center
  2725 Binion Road, Apopka, FL 32703-8504
- **E-mail:** djg@ufl.edu

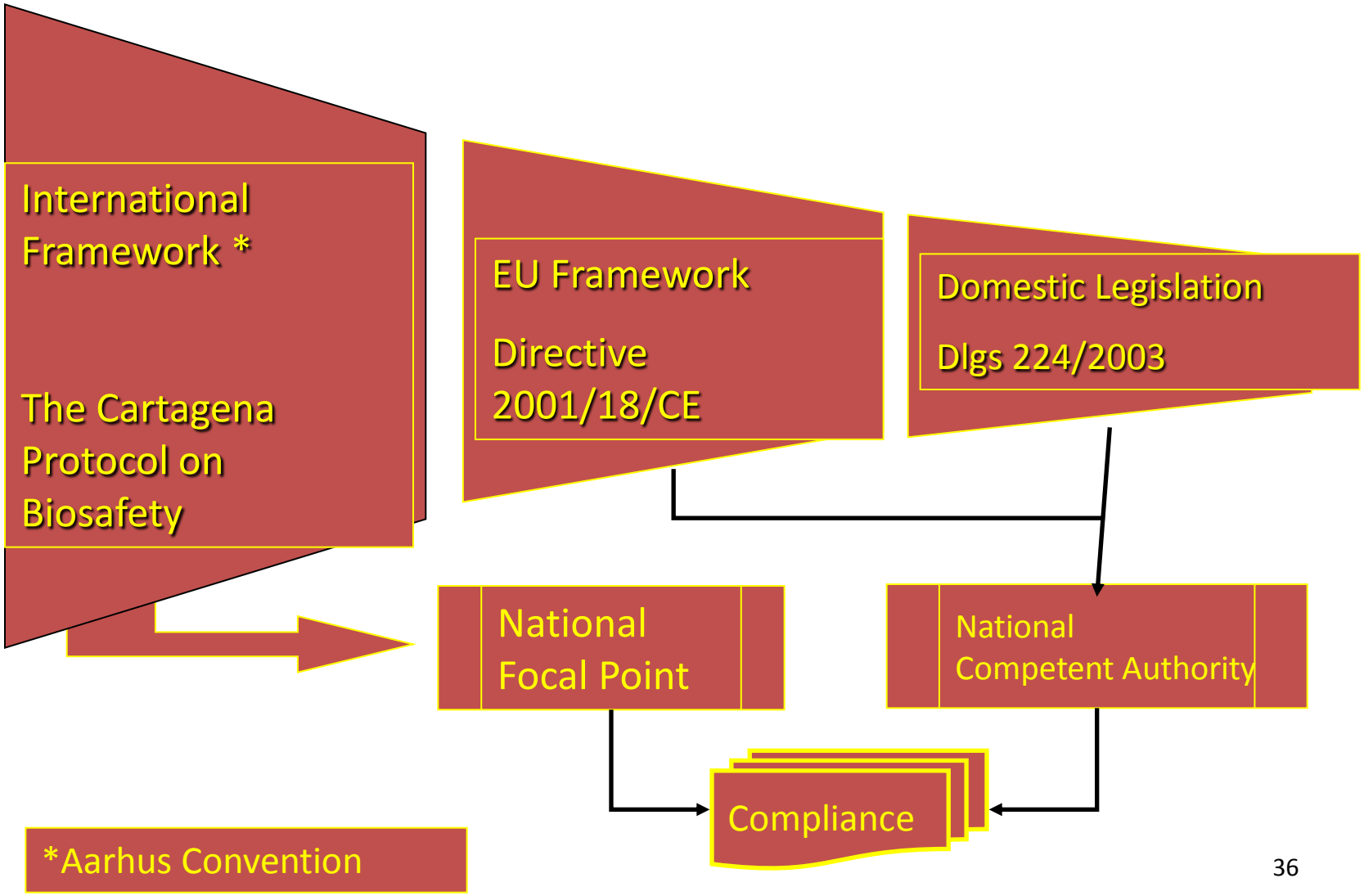
Why we need the open field trials with GM plants:



Which risks: environment?, Human Health?, the agricultural economy????

Which benefits: increased production efficiency and quality

The Biosafety legal framework
UNIDO-UPM E-BIOSAFETY MASTER



The European Commission adopted Directive 2001/18/EC (repealing Directive 90/220/EEC) to govern the deliberate release of GMOs into the environment.

- Risks for human heath
- Risks for the environment
- Risks for agriculture systems

- **'CASE BY CASE'**

(Gene and Plant Specie)

- **'ON SCIENTIFIC BASE'**

- Eliminate all the antibiotic markers with possible risks to human health and environment, followed by the EFSA opinion on the type of antibiotics (*nptII*).

GMO Risks and Benefits evaluation
EXPERIMENTAL TRIALS
INTERACTION OF COMPETENCES

- Gene function in the donor organism.
- Gene effect on the phenotype.
- Evidence of toxicity and allergenic effects.
- Persistency and invasiveness in agriculture.
- Impact on no target organisms.
- Gene flow (soil-microrganisms, environment-plant)

Decree No. 224 has now been completed by the recent **Decree No. 5 (28/01/05)**, prepared by the **Minister of Agriculture**, that identified for the first time in Italy the major rules of **co-existence** between GMO and traditional cultivation systems (**Regions acceptance within June 2006**).

UNICO ESEMPIO DI STUDIO METABOLOMICO MEDIANTE NMR IN VITE GM

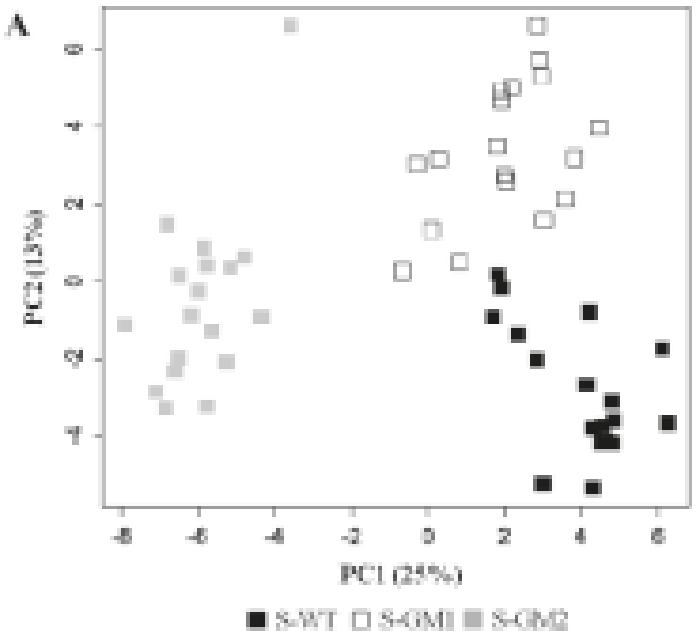


Figure 2. Silcora sample multivariate analysis. (A) Score plot obtained by application of PCA on mean-centered and scaled spectral bins recorded on berry extracts of the Silcora cultivar. The first two PCs explain 25% (PC1) and 13% (PC2), respectively, of the total variance. (B, C) Loading plots for spectral bins, along PC1 and PC2, respectively. The black bins labeled with numbers are described in the text as representative of meaningful bins responsible of separation along the respective PC dimension.

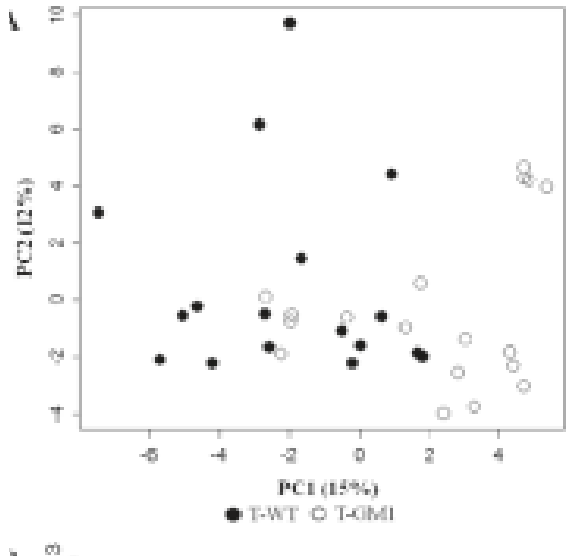


Figure 4. Thompson samples multivariate analysis. (A) Score plot obtained by application of PCA on mean-centered and scaled spectral bins recorded on berry extracts of Thompson cultivar. The first two PCs explain 15% (PC1) and 12% (PC2), respectively, of the total variance. (B, C) Loading plots for spectral bins, along PC1 and PC2, respectively. The black bins labeled with numbers are described in the text as representative of meaningful bins responsible of separation along the respective PC dimension.

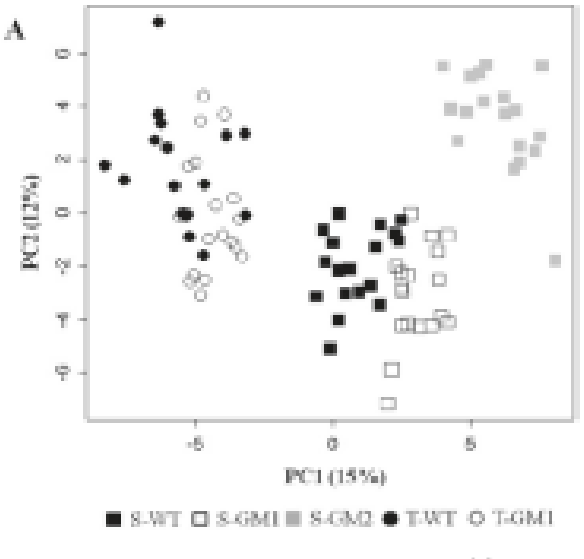


Figure 6. All grape sample multivariate analysis. (A) Score plot obtained by application of PCA on mean-centered and scaled spectral bins recorded on berry extracts of all cultivars and genotypes. The first two PCs explain 26% (PC1) and 12% (PC2), respectively, of the total variance. (B, C) Loading plots for spectral bins, along PC1 and PC2, respectively. The black bins labeled with numbers are described in the text as representative of meaningful bins responsible of separation along the respective PC dimension.

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Unsupervised Principal Component Analysis of NMR Metabolic Profiles for the Assessment of Substantial Equivalence of Transgenic Grapes (*Vitis vinifera*)

Gianfranco Picone,[†] Bruno Mezzetti,[§] Elena Babini,[†,*] Franco Capocasa,[§] Giuseppe Placucci,[†] and Francesco Capozzi[*,†,*]

[†]Department of Food Science, University of Bologna at Cesena, Piazza Goidanich 60, 47520 Cesena (FC), Italy

[§]Department of Environmental Science and Vegetal Productions, Marche Polytechnic University, Via Brecce Bianche, 60131 Ancona (AN), Italy

[*]Centre of Magnetic Resonance (CERM), University of Florence, Via L. Sacconi 6, 50019 Sesto Fiorentino (FI), Italy

CONCLUSIONS

•First example of trasformation of Corvina cultivar (after confirmation by Southern Blot analysis) with an anviral construct

•Obtainement of transgenic lines- prototype for future virus resistance assessment

•Obtainement of transgenic lines for studyng AUCSIA function during grape berry growth

WORK in progress and future….

• **Virus infection experiments on *Nicotiana benthamiana* plants and grape to test the efficiency of the hpconstruct for multiple resistance**

• Production of Pinot noir and 1103Paulsen transformed with the hpVirusesGFLV-GLRaV construct

• Use the transgenic rootstock lines to test the possibility of conferring virus •resistance to scion.

Università di Verona
Dipartimento di Biotecnologie

Dott.ssa Tiziana Pandolfini
Dott.ssa Annalisa Polverari
Dott.ssa Barbara Molesini
Dott.Youry Pii

Università Politecnica delle Marche
Dipartimento di Scienze Agrarie, Alimentari ed Ambientali (D3A) Ancona

Prof. Bruno Mezzetti
Dott.ssa Silvia Sabbadini

Dott. Oriano Navacchi
Vitroplant Technologies for Agricultural plants, Cesena

A GM GRAPE e is still missing on the market.

It can be a consumer Right but also of high Benefit for the growers.