Improving Wine and Table Grape Cultivars Using Precision Breeding and Genome Editing

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Crop Improvement in Grapevine

- World's most valuable fruit crop
- United States – 10th most valuable agricultural crop
- Job creation – 1.7 million jobs
- National economic impact – $ 219 billion annually Wine America (National Association of Wines), 2017
- Maryland grape industry – 1,000 acres, 84 wineries, 2.3 million bottles of wine, annual economic contribution – $ 47 million

Limitations of Conventional Breeding for Grape Improvement

- Extreme heterozygosity
- F1 hybrid produced is of intermediate quality
- Incompatibility barriers between *Vitis* species

Precision Breeding (PB)

An approach to plant genetic improvement that transfers only specific traits among sexually compatible species via the relatively stable *mitotic cell division pathway* in order to avoid the significant genetic disruption imposed upon conventional breeding by meiosis

Gray et al. 2016. Acta Hort., 1115: 49-56

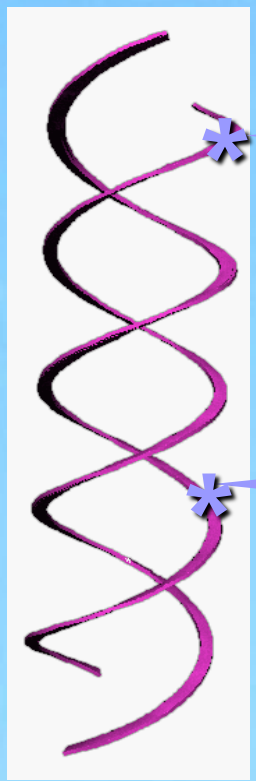
Cultivars obtained through PB will not be regulated by USDA, Release # 007018

Factors Enabling Precision Breeding of Grapevine

- Advances in genome sequencing technology and availability of the *Vitis* genome
- Grape cell culture and regeneration system
- Gene insertion system to insert traits of interest and recover PB vines

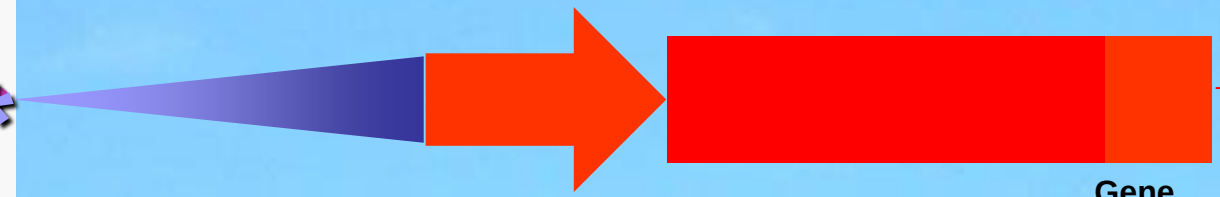
Precision Breeding (PB) Technology

Major difference is in the ***source*** of DNA sequences inserted in plants



Grape Chromosome

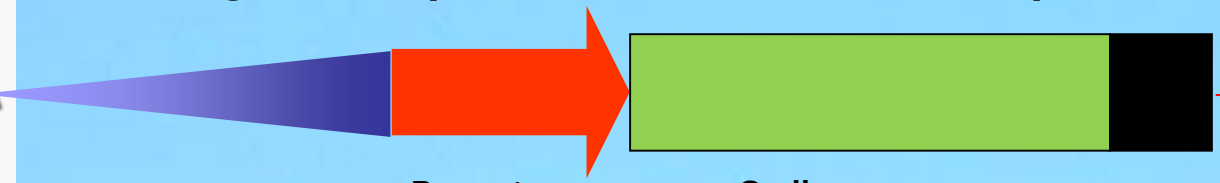
Cisgenic sequence - Native unaltered sequences



Promoter **Coding sequence** **Gene Terminator**

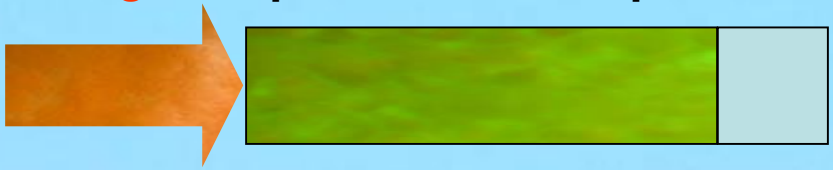
PB

Intragenic sequence – Native altered sequences



Promoter (Chr. 2) **Coding sequence (Chr. 19)** **Gene Terminator (Chr. 7)**

Transgenic breeding – sequences across plant/animal kingdom



Viral Promoter

Insect coding sequence

Bacterial Terminator

Optimizing Precision Breeding Technology

- Refine grapevine cell culture and plant regeneration systems

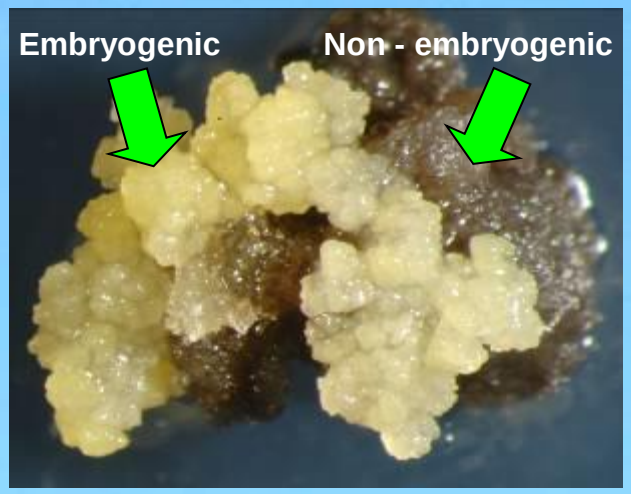
- Optimize gene insertion systems

- Identification and screening of grape-derived genetic elements and genes for trait improvement

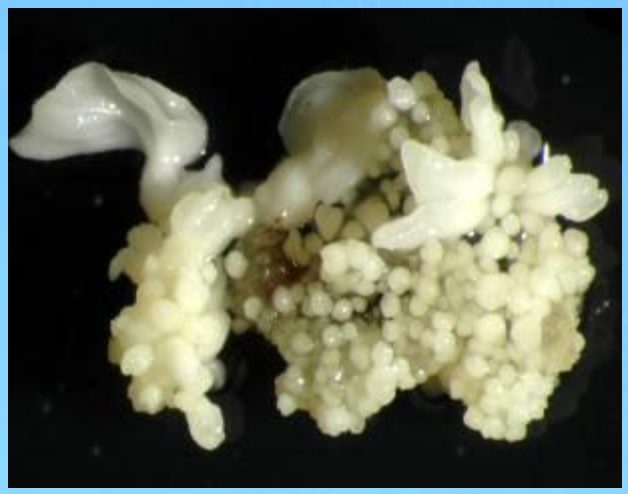
Grape Embryogenic Cell Culture System

Initiation from leaves and flower tissues

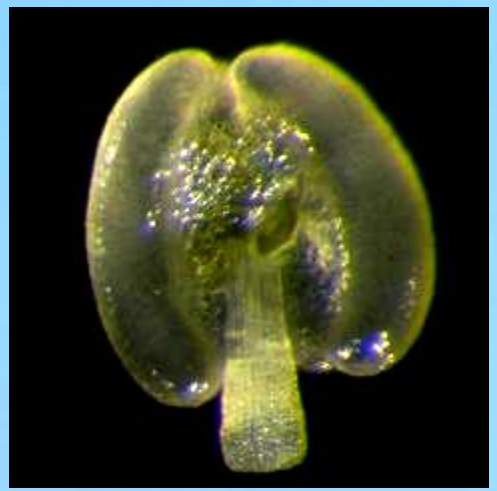
Leaf explant



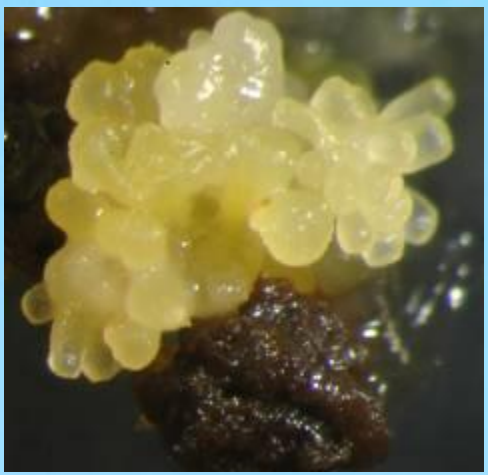
Embryogenic callus



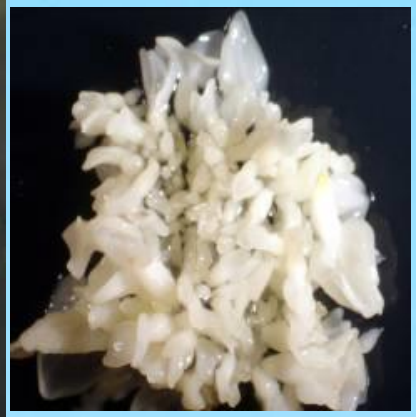
Embryo development



Flower explant



Embryogenic callus



Embryo development

Embryo germination

Gene Insertion System

Embryo explants

+

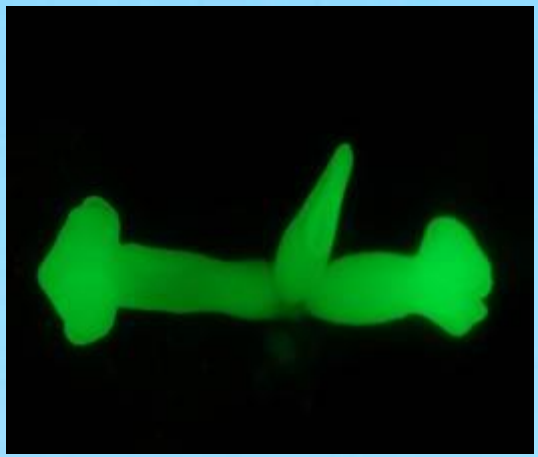
Agro culture

Co-cultivate
48 h

Wash explants

Callus induction
Medium (DMcck)

Embryo development
Medium (X6cck70)



Select transgenic embryos

Regenerate plants

GFP Expression in Muscadine Grape ‘Alachua’

(Dhekney et al. 2008, Plant Cell Reports, 27, 865-872)

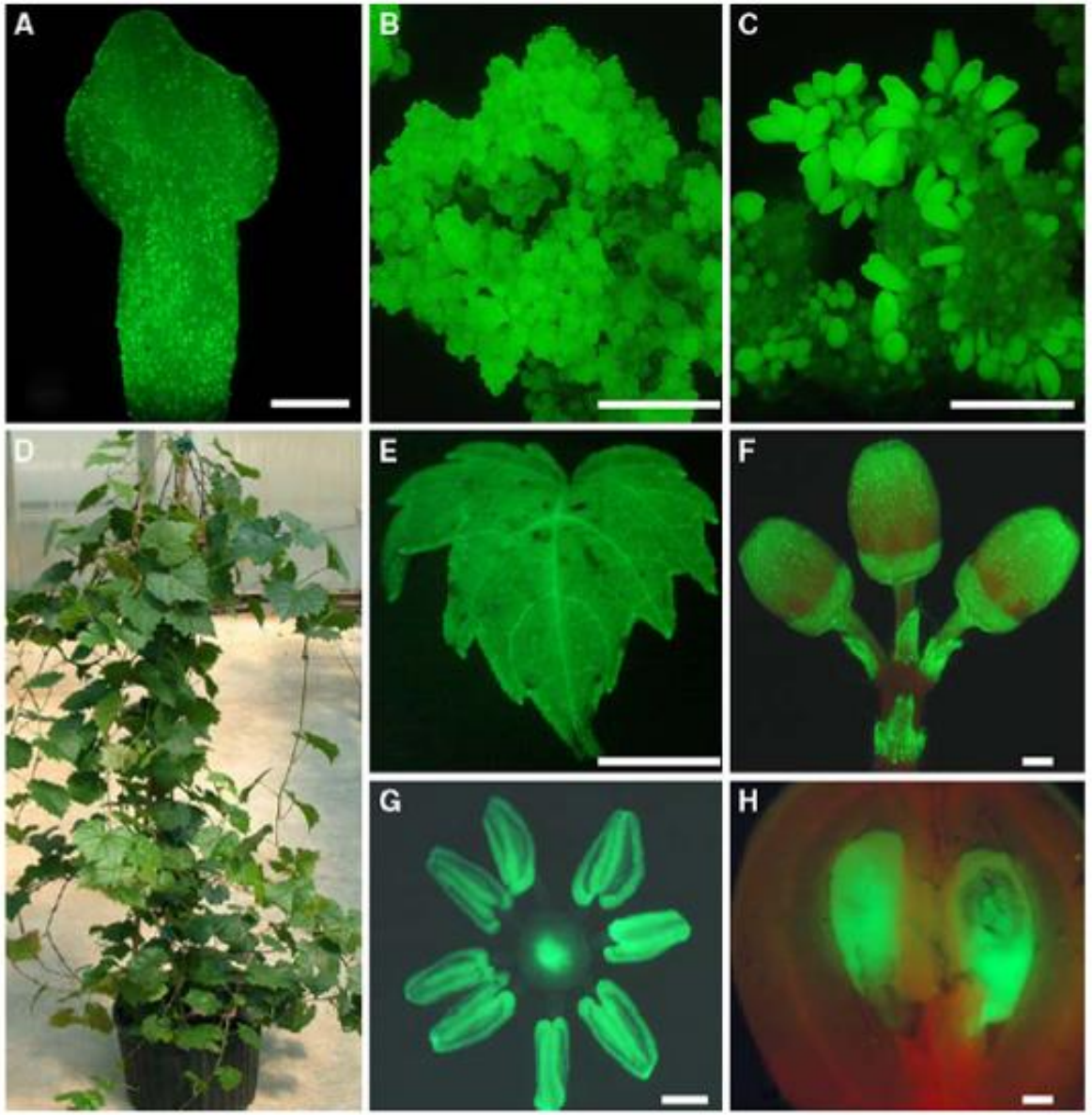
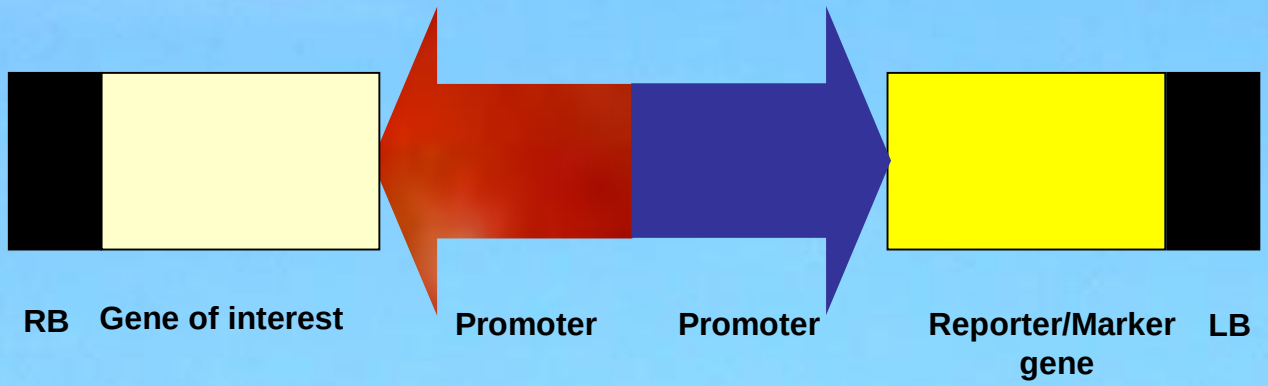


Fig. 2 Production of transgenic plants of *V. rotundifolia* "Alachua". **a** Transient GFP expression in SE explants 4 days after co-cultivation with *Agrobacterium*. **b** GFP expressing transgenic callus and PEM 60 days after co-cultivation. **c** Transgenic secondary SE expressing GFP 90 days after co-cultivation. **d** A true to type transgenic "Alachua" plant in the greenhouse. Uniform GFP expression in mature leaf (**e**), inflorescence (**f**) and anthers (**g**). **h** GFP expression in developing berry. Note that the berry exocarp and mesocarp, which is devoid of GFP, emits red chlorophyll-induced autofluorescence whereas the embryo and endosperm show uniform GFP expression. *Scale bars*: Fig. **a**, **f**, **g**, **h** = 0.5 mm, Fig. **b**, **c**, **e** = 5 mm

Isolation of Grape-Derived Genes and Regulatory Elements



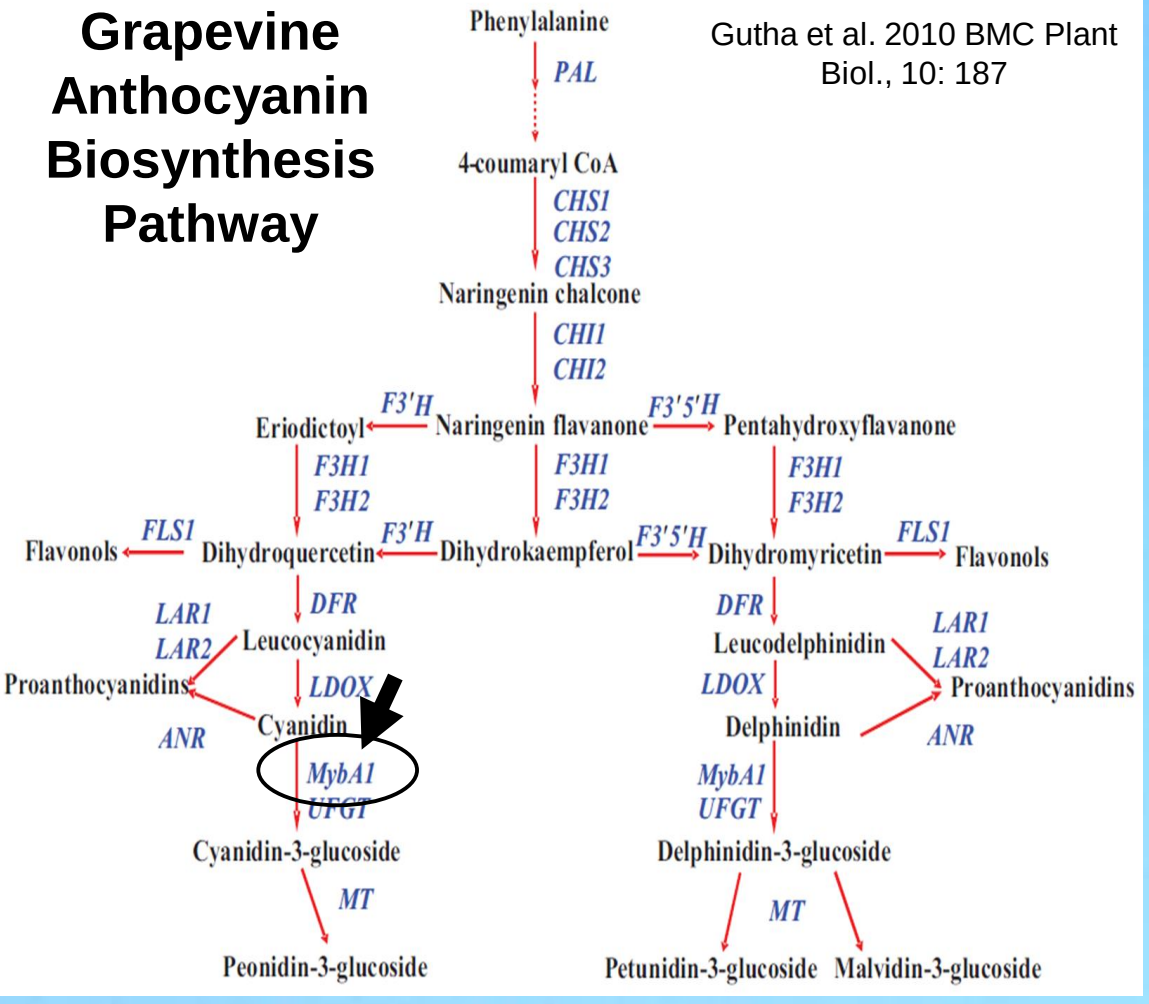
Schematic Representation of a Gene Cassette

- First Generation GM crops (corn, soybean, canola, etc.) – grown on > 93% of acreage in the United States

Reporter gene – jelly fish or E.coli (GFP, GUS)
Promoters – virus (CaMV35S)
Selectable marker – E. coli (NPTII)
Genes conferring traits of interest – bacteria, viruses
Border sequence (RB/LB) – bacteria (*Agrobacterium*)

Development of Grape-Derived Reporter Gene System

Grapevine Anthocyanin Biosynthesis Pathway



Gutha et al. 2010 BMC Plant Biol., 10: 187

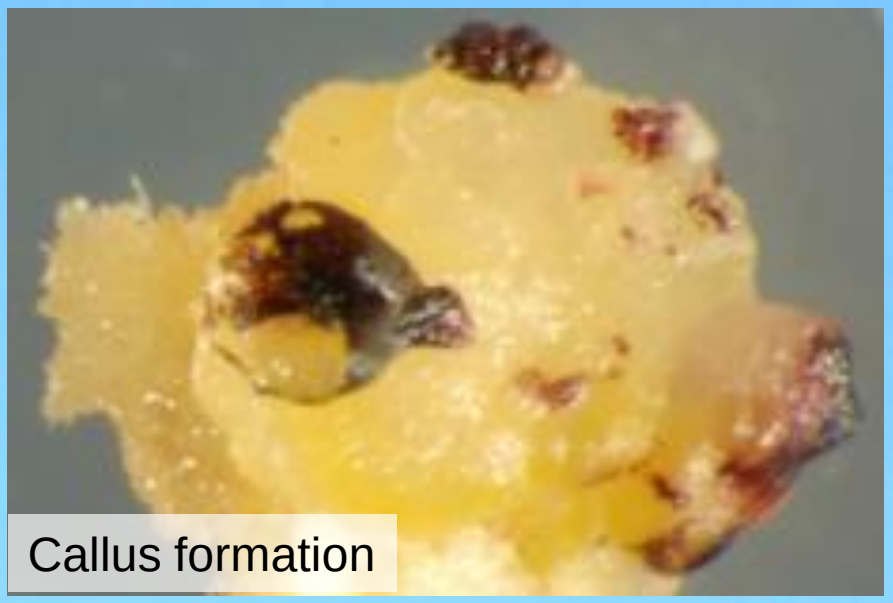
Why VvMybA1 ?

- **Regulates anthocyanin biosynthesis**
- **Visual indicator; easily detectable**
- **Ability to determine gene insertional events in a non-destructive, real-time manner.**
- **Species-specific**

VvMybA1 +

VvMybA1 -

MybA1 Gene Expression in Grape Tissues



Li, Z., Dhekney, S. & Gray, D. 2011. Trans Res., 20: 1087-1097

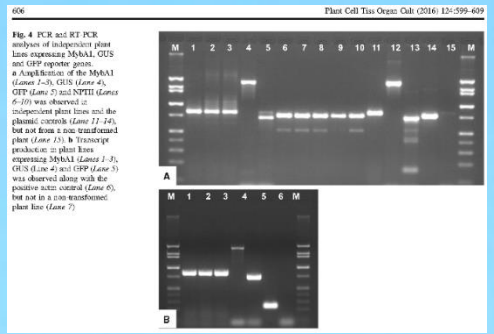
Grapevine Lines Expressing MybA1

Table 1 Influence of GFP, GUS and *Vv*MybA1 reporter genes on transient and stable gene expression, and transgenic embryos recovery in *Vitis*

Reporter gene	Transient expression (%)[1]	Stable callus formation (%)	Transgenic embryo lines (%)
Thompson Seedless			
GFP	80.0[b]	51.1[b]	8.9[a]
GUS	86.8[a]	57.8[a]	9.6[a]
*Vv*MybA1	89.4[a]	41.2[c]	8.0[a]
Bronx Seedless			
GFP	72.2[b]	52.2[b]	8.1[a]
GUS	65.0[c]	61.6[a]	8.7[a]
*Vv*MybA1	95.5[a]	46.4[c]	4.4[b]

Data for each treatment represents the mean of three replicates, each replicate consisting of at least 30 SE. SE with at least 10 GFP or GUS positive cells 3 days after *Agrobacterium* treatment were scored as positive. For the *Vv*MybA1 reporter gene, explants exhibiting at least 10 red spots were scored as positive. SE with at least one proliferating red pigmented, GFP or GUS positive callus after 8 weeks and a SE line after 12 weeks were scored as positive

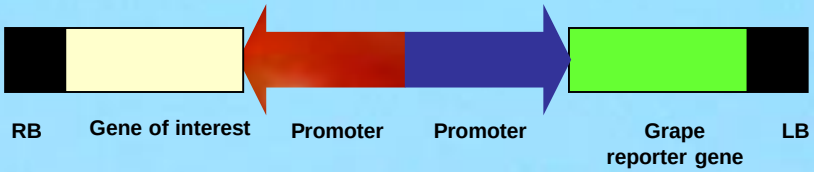
[1] Statistical analysis was carried using Proc GLM and ANOVA procedures of SAS. Means within a column represented by the same letter were not significantly different according to Student–Newman–Keuls test ($\alpha = 0.05$)



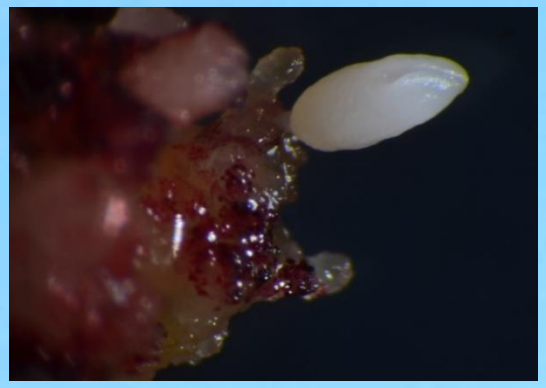
Molecular analyses

Kandel et al. 2016, Plant Cell Tiss. Org. Cult., 124: 599-609.

Kandel et al. 2016. Acta Hort., 1115: 57-61

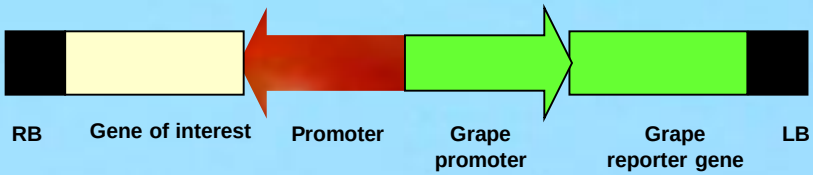


Cell Culture-Specific MybA1 Expression Using an Embryo Specific Promoter



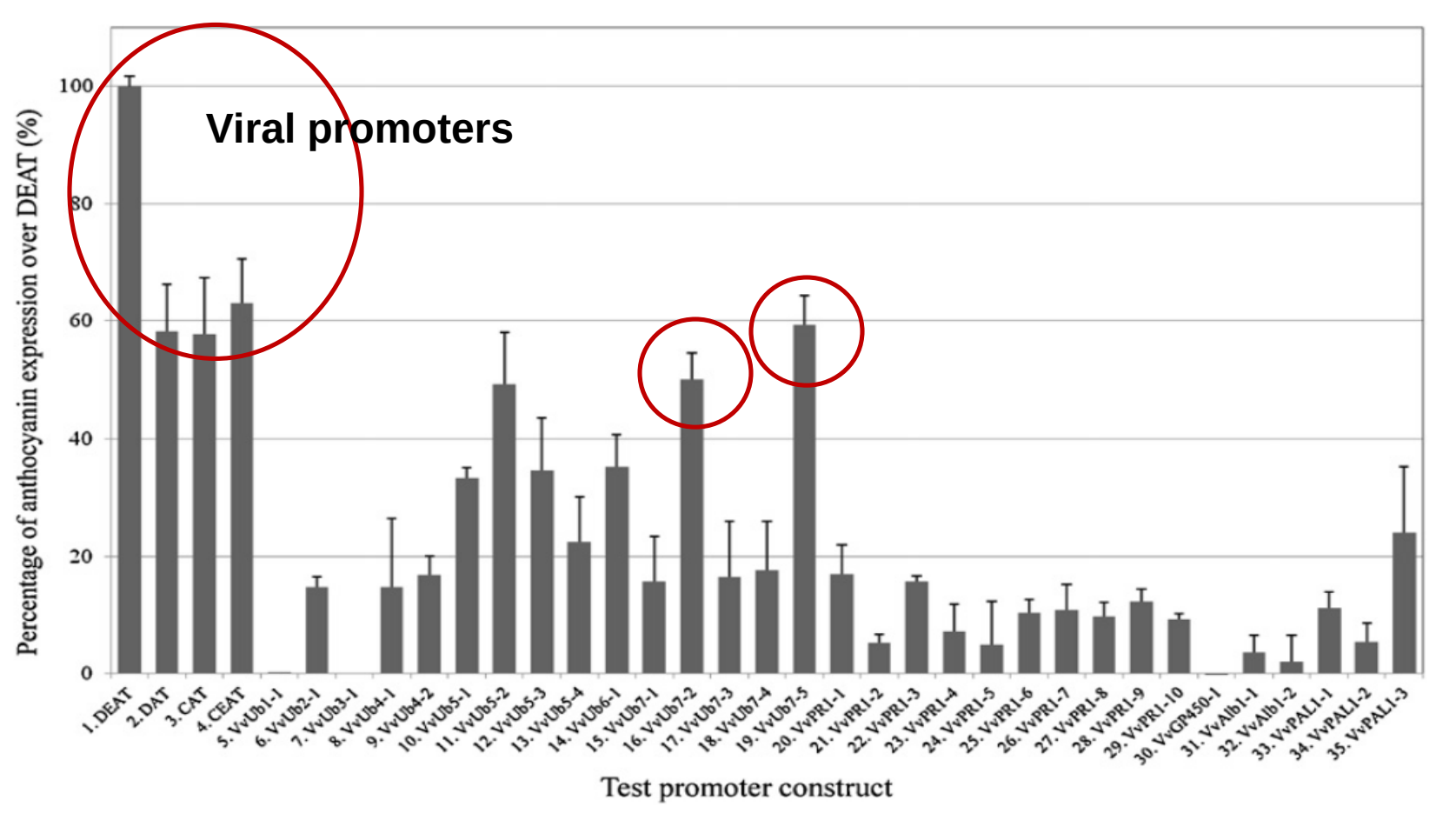
Control 2S albumin 35S

- VvMybA1 was fused to a VvAlb1 (2S albumin) promoter
- Anthocyanin expression is observed in tissue culture but not in regenerated plants

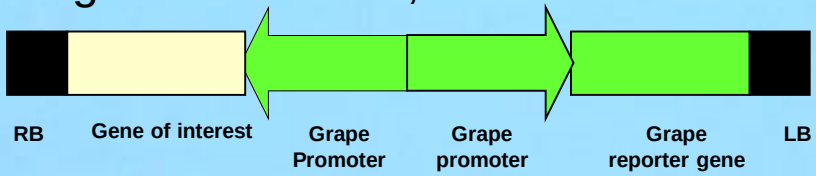


Kandel et al. 2015. HortSci, 50 (9):S370

Isolation of Grape-Derived Promoters



VvUb7-2 and VvUb7-5 exhibit gene expression comparable to a single 35S promoter
Li et al. 2012, Transgenic Research, 196: 132-142 – **collaborator data**



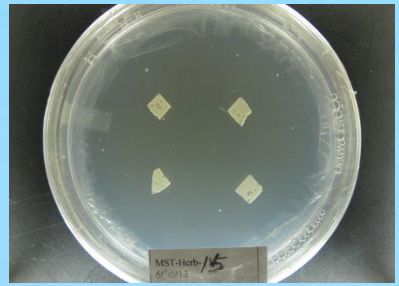
Development of Grape-Derived Selectable Marker System

- *Acetolactate Synthase* (ALS) controls amino acid biosynthesis and is a herbicide target
- Isolation and testing of a grapevine ALS gene
- Inserted VvALS2-F2 mutant in tobacco cultures
- Currently testing the marker in grape

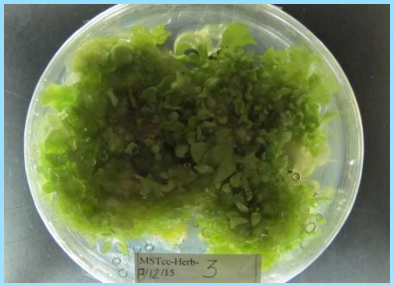
Monument

(1 mg/L)

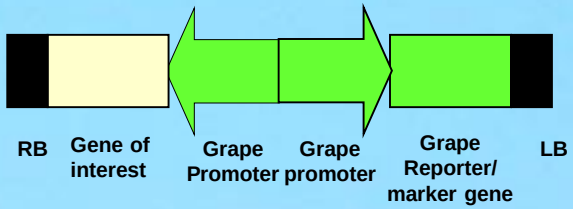
Label: 66 mg/L



Control



Herbicide resistant



Functional Genes for Traits of Interest

- Grape genome – No. of proteins produced in response to pathogen attack (Pathogenesis Related) or abiotic stress

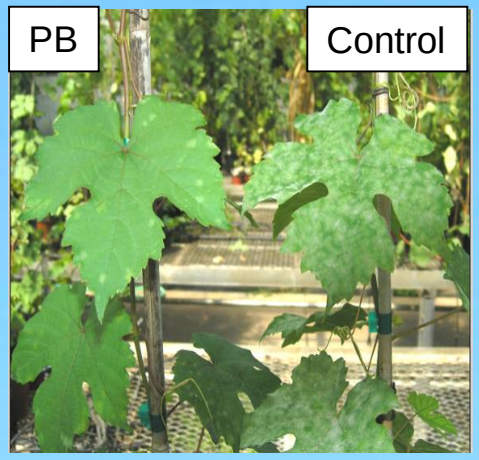
- Some proteins – Impart biotic/abiotic stress tolerance (drought stress)

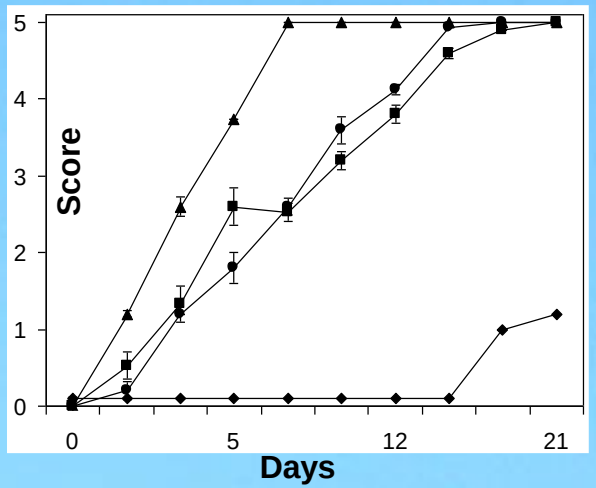
  (Eg. Thaumatin-like proteins, Stilbene synthase, VPP1)

- Proteins – obtained from sexually compatible wild species for incorporating abiotic/biotic stress tolerance in commercial cultivars

Using *Vitis*-derived VVTL-1 for Improving Disease Resistance

Greenhouse screening





Score (No. of lesions per leaf)

1 = no lesions, 2 = <10 lesions, 3 = 10-15 lesions, 4 = 15-20 lesions, 5 = >20 lesions

Table 1. Estimation of copy number in VVTL-1 plant lines using quantitative real-time PCR

No.	Sample name	Mean CP [a]	STD CP [b]	Mean conc. [c]	STD conc.	Estimated copy no.
1	Plasmid- 1 copy	26.7290	0.0944	1.0115	0.0318	1
2	Plasmid- 2 copies	24.8840	0.0488	1.9740	0.0604	2
3	Plasmid- 3 copies	23.5178	0.0579	3.0514	0.0247	3
4	Plasmid- 4 copies	21.2204	0.0133	4.0783	0.0546	4
5	Plasmid- 5 copies	20.7834	0.0241	4.9732	0.0249	5
6	TV3	24.9649	0.0488	1.8550	0.0310	2
7	TV5	27.0436	0.0857	0.9780	0.0265	1
8	TS-Ck[d]	32.7319	0.5677	0.1299	0.0338	0

[a] Average values of crossing points (cp) from three sample replicates

[b] Standard deviation values for crossing points

[c] Average DNA concentration values extrapolated from cp values

[d] Sample values for non-transformed control *V. vinifera* "Thompson Seedless"

Copy no. analysis

Table 2. Amount of cisgenic VVTL-1 protein produced in engineered and control grapevines

ELISA samples	Absorbance values (405 nm) [a]	Protein produced (μg 100 μl^{-1}) [b]	Protein produced (μg g^{-1} leaf tissue)
Pure protein (μM)			
0.0	0.0700	0.002	
0.01	1.8640	2.434	
0.05	1.9420	2.568	
Plant samples			
TS-Ck	0.0710	0.003	0.0695 c
TV3	0.0920	0.027	0.5439 b
TV5	0.1570	0.101	2.0222 a

Analyzing protein expression using ELISA

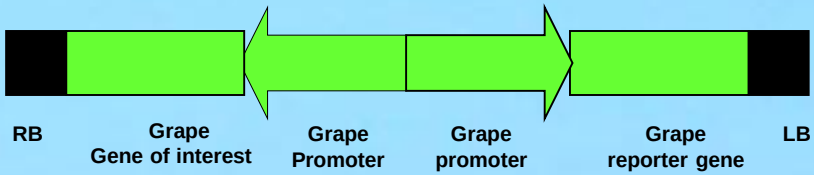
Field Testing of PB Grapevines

Control vine

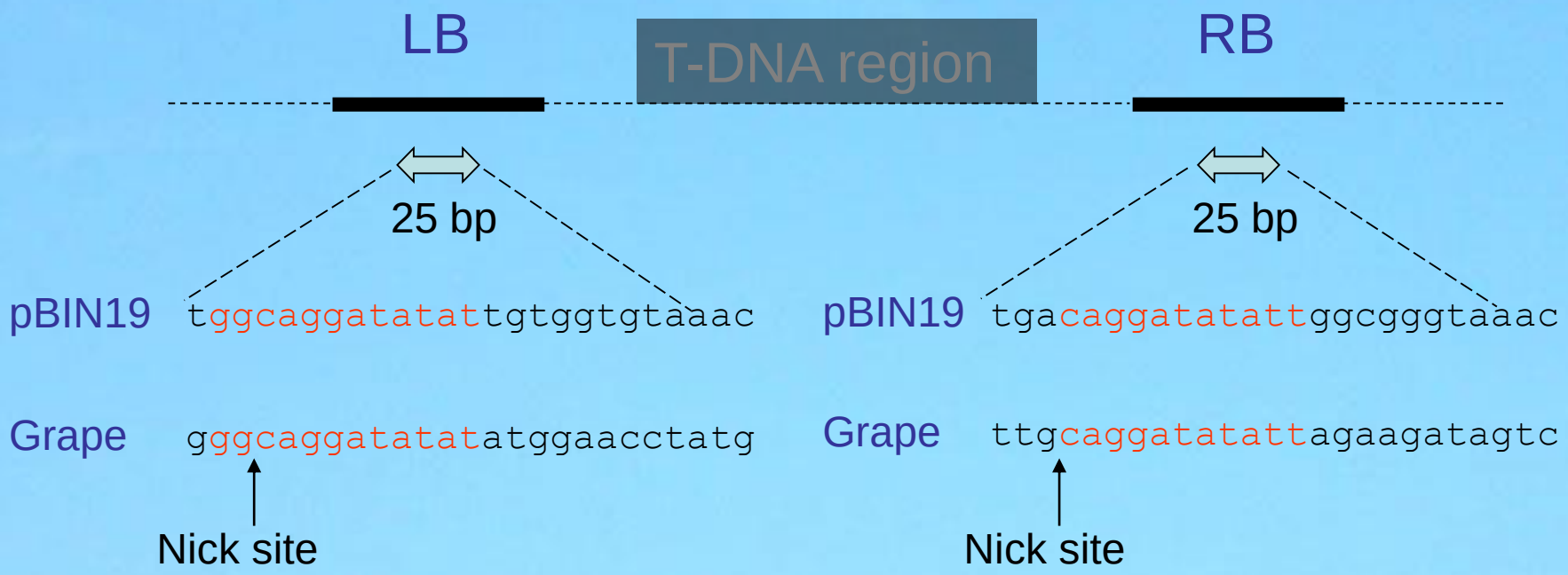
PB vine

Dhekney et al. 2011. In Vitro Cell. Dev. Biol., Plant: 47: 458-466

PB vines exhibited significantly lower development of black rot compared to non-transformed control vines



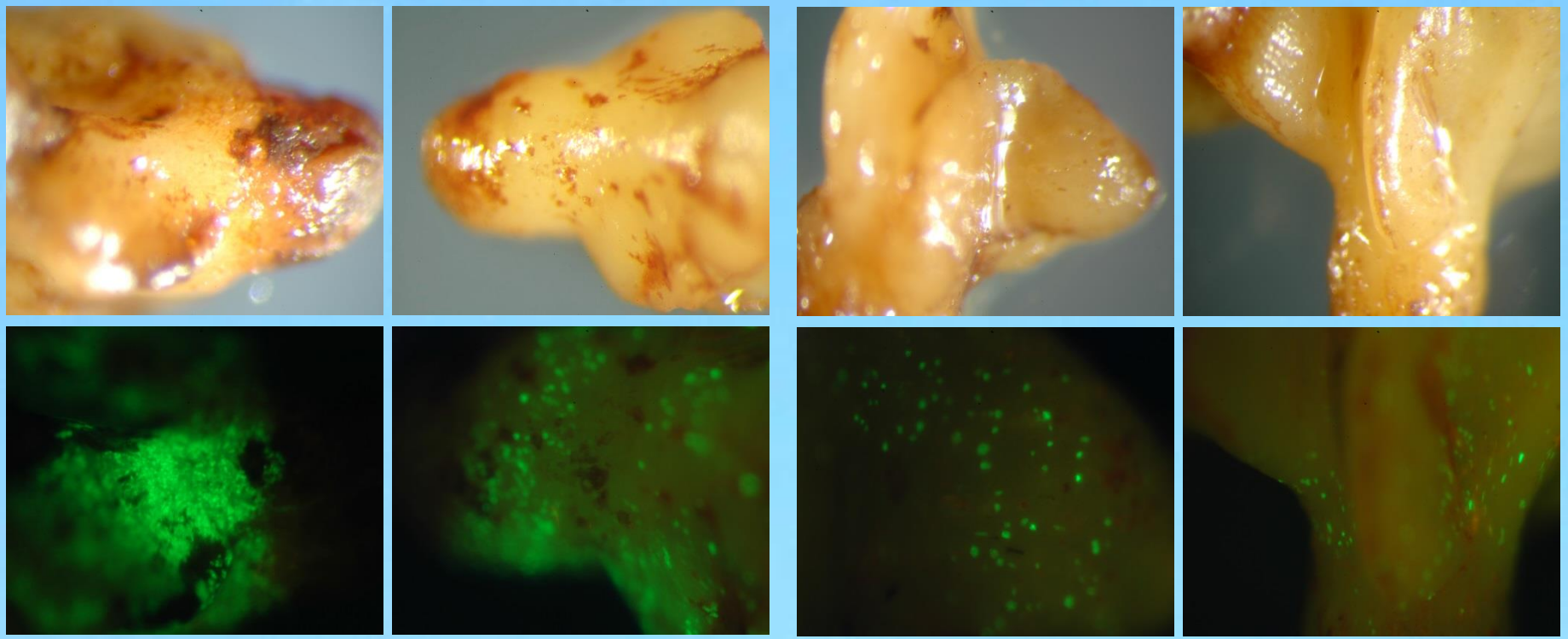
Border Sequences and Cleavage Sites Derived from *Agrobacterium* and Grapevine



Sequences homologous to *Agrobacterium* borders can be found in the grape genome

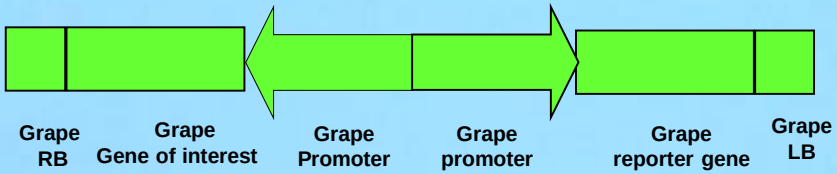
Gene Expression using *Agrobacterium* and Grape-Derived Borders

GFP Expression in Somatic Embryos with Constructs harboring Grape Borders



Agrobacterium border

Grape border



Implementation of Precision Breeding

Grapevine as an Example

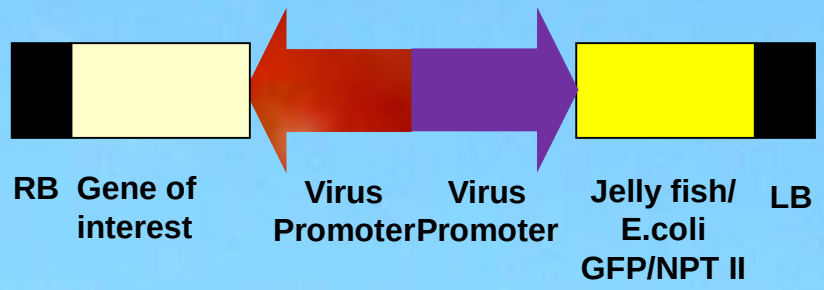
Grapevine cell culture and transformation system

Grape-based Reporter gene and promoter

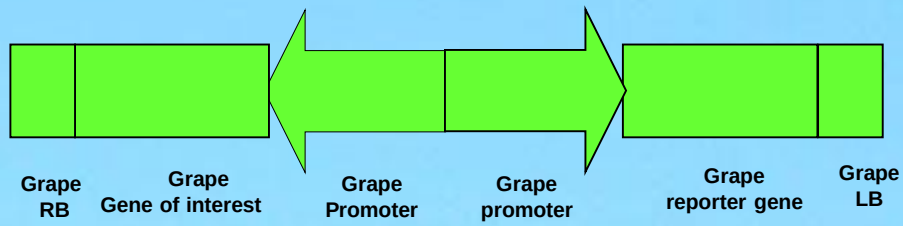
Grape-based ALS selectable marker

Functional gene/s for trait improvement

Transgenic Gene Cassette

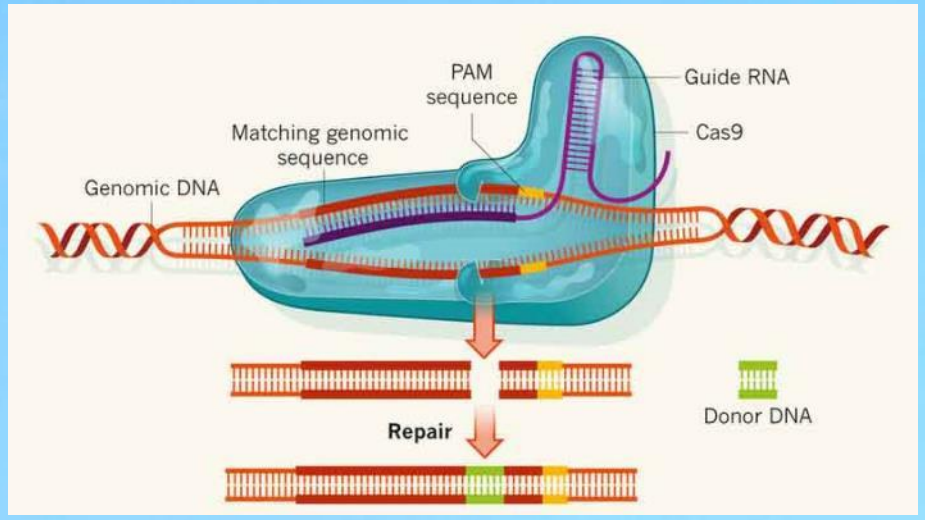


Precision Bred Gene Cassette



Genome Editing for Crop Improvement

Using CRISPR/Cas9/Cms1 for genome editing



Carr, 2017. Global Biotech. Insights

Genome Editing Pipeline

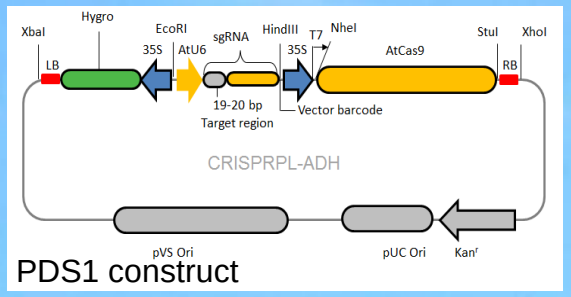
Grapevine cell culture and transformation system

Grape-based Reporter gene and promoter

ALS-based selectable marker

Target traits for deletion/expression

Genome Editing – Proof of Concept



PDS1 construct


somatic embryos


Agro


Callus lines


embryo lines


Edited germinated line


Control germinated SE


Edited and control shoots


Edited and control plants


control leaf

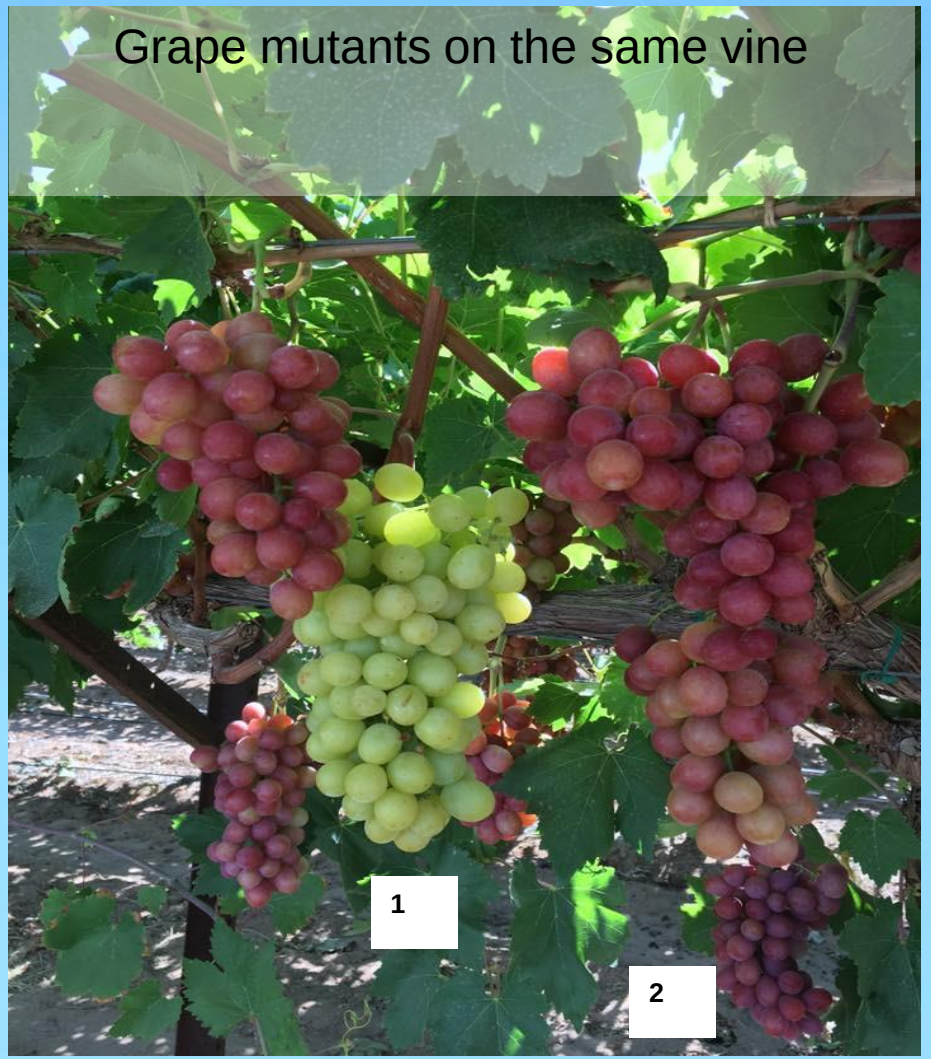

Edited leaves

- Leaf color can be modified by knocking out PDS 1 gene (involved in carotenoid biosynthesis)
- Knock out – loss of chlorophyll resulting in totally albino plants or mosaic plants

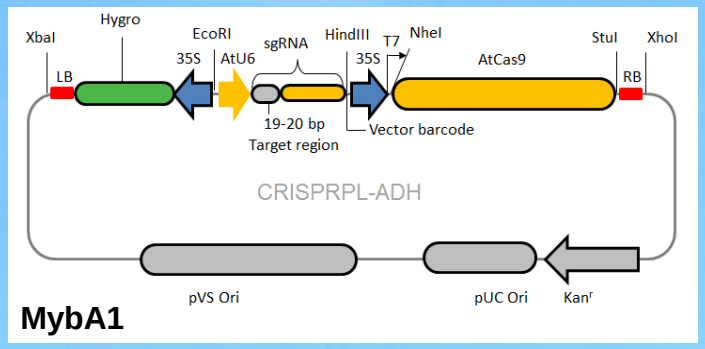
Modifying Berry Color Using CRISPR-Cas9

Commercial Applications

- Rapid change berry color in existing commercial cultivars
- Dramatically reduce the time required to get a new cultivar (*8-10 years* for breeding compared to *2-3 years* by genome editing)
- Green versions of all red cultivars and vice versa



Genome Editing for Color Traits



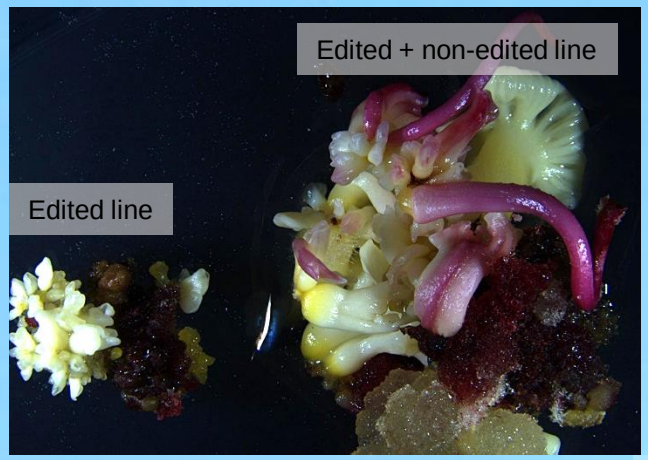
CRISPR construct

Normal somatic embryos

SE expressing MybA1

+

Agro culture



Non-edited phenotype

Edited Mutant Phenotypes

- VvMybA1 expressing embryos were transformed with a CRISPR construct harboring a gRNA for VvMybA1
- Following transformation, reversion of phenotype is observed
- Commercial applications – rapidly change color in cultivars

Commercial Implications of Precision Breeding and Genome Editing

An official website of the United States government Here's how you know

USDA U.S. DEPARTMENT OF AGRICULTURE

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Secretary Perdue Issues USDA Statement on Plant Breeding Innovation

(Washington, D.C., March 28, 2018) – U.S. Secretary of Agriculture Sonny Perdue today issued a statement providing clarification on the U.S. Department of Agriculture's (USDA) oversight of plants produced through innovative new breeding techniques which include techniques called genome editing.

Press Release
Release No. 0070.18

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Under its biotechnology regulations, USDA does not regulate or have any plans to regulate plants that could otherwise have been developed through traditional breeding techniques as long as they are not plant pests or developed using plant pests. This includes a set of new techniques that are increasingly being used by plant breeders to produce new plant varieties that are indistinguishable from those developed through traditional breeding methods. The newest of these methods, such as genome editing, expand traditional plant breeding tools because they can introduce new plant traits more quickly and precisely, potentially saving years or even decades in bringing needed new varieties to farmers.

What will not be regulated?

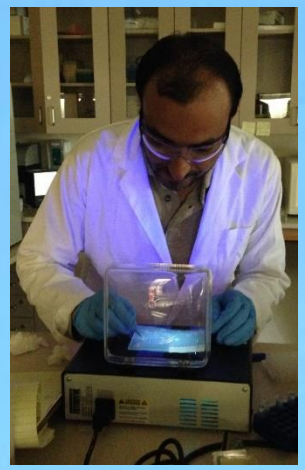
- Deletions
- Single base pair substitutions
- Insertions from compatible plant relatives
- Complete Null Segregants

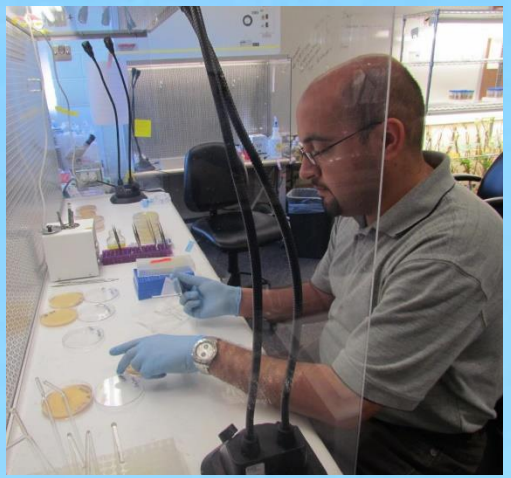
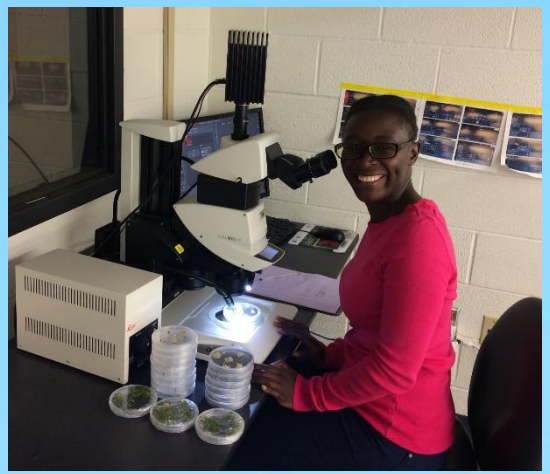
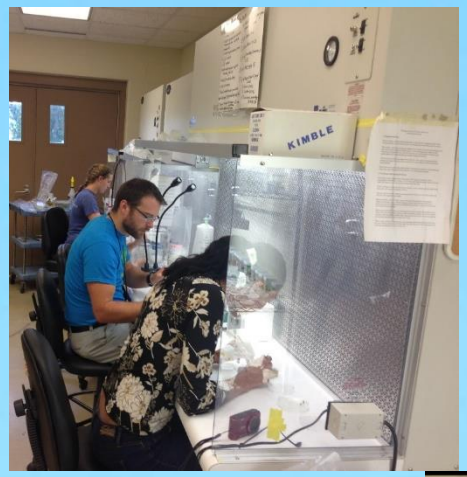
Target Traits for Genome Editing *(research in progress)*

- Improved flowering – knock out TFL 1 gene
- Reduced acidity – knock out idonate dehydrogenase gene
- Powdery mildew resistance – knock out MLO (mildew locus O) receptor gene
- Drought tolerance – inserting VPP-Gr. 1 genes
- Optimize CRISPR-Cas9 system using *ribonucleoprotein (RNP)* complex for transient expression

Students, Post-Docs, Visiting Scientists

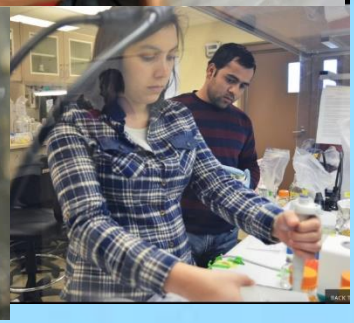








Acknowledgements

Collaborators

- US – University of Florida, Washington State University, Fort Valley State University, University of Nevada
- International – University of Guelph – Canada, Hebei Agricultural University – China, Ain Shams University and Cairo University – Egypt, Delle Marche Polytechnic University - Italy

Funding Agencies

- USDA (T-STAR, International Programs, BRAG, SCRI - block grant program, Borlaug), NIH-INBRE, WY Dept. of Ag., Sun World International

University of Maryland Eastern Shore – Ag. Experiment Station