TECHNOLOGICAL IMPLEMENTATIONS TOWARD SUCCESSFULL APPLICABILITY OF MARKER-FREE GENOME EDITING IN *VITIS VINIFERA*

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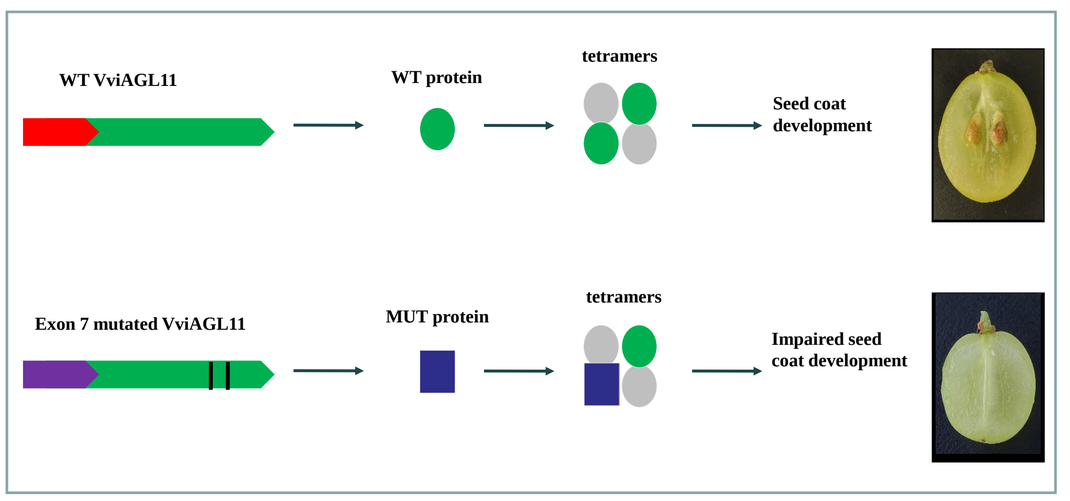
Background and Aims

New Plant Breeding Techniques (NPBTs) protocols have been developed to produce new grape varieties with improved quantitative and qualitative characteristics, modifying only target genes and, than, keeping intact all the other typical characteristics of the variety. The applicability of these NPBTs is strictly dependent to some main factors: to identify the genes involved in interesting traits, the availability of reliable transfection protocols, the availability of reliable regeneration protocols and the improvement embryogenic competent callus formation from recalcitrant genotypes .
In order to perform marker free editing, we present the recent advancement of our research team in develop genomic tools for the application of next generation molecular breeding in *Vitis vinifera* L. By combining genomic and trascriptomics we were able to in deep study some candidate genes important in grape breeding and use these informations to precise design of gRNAs/CAS modules to target specific sites of mutation. To fine-tune the whole procedure, we chose to target VviAGL11, the gene that induces seedlessness in table grapes.
Grape cultivars: Victoria, Red Globe, Italia and Thompson.



Simplified model of VviAGL11 causative effect on stenospermocarpy

Results

We optimized methods to improve the somatic embryogenesis ability of recalcitrant genotypes, such as the Italia cultivar. Indeed, in grapes a wide application of NPBTs in Vitis is hindered by the null or very low aptitude to generate embryogenic calluses of many important varieties. By testing different protocols, we observed that besides the strong genotype influence, in our system the formation of total and embryogenic calluses was influenced by the type of sterilization and the cultivation substrate. Our modified protocol increases the production of embryogenic calluses, which was a fundamental aspect for the applicability of NPBTs such as genome editing.

Immature inflorescences cv Italia

5 months

Embryogenic callus cv Italia

2020

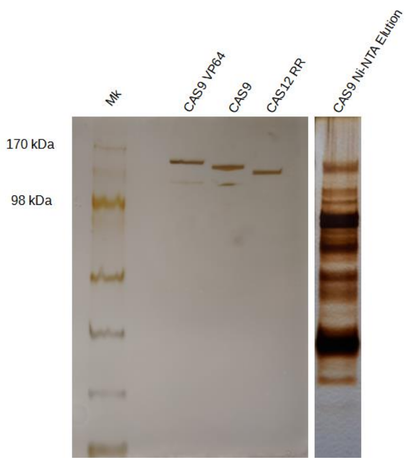
Cultivar	Embryogenic Callus (%)
Aglianico	2,1
Autumn Royal	2,4
Chardonnay	16,9
Crimson	6,2
Glera	0,7
Italia	0,4
Negromaro	0,5
Palieri	0,5
Primitivo	0
Red Globe	2,1
Thompson	10,5
Summer Royal	3,8
Victoria	2,1

2021

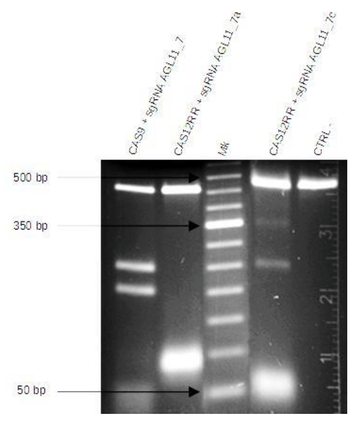
Cultivar	Embryogenic Callus (%)
Chardonnay	28,8
Italia	0,86
Nero di Troia	0,19
Primitivo	0,36
Red Globe	3,06
Thompson	38,18
Victoria	2,86

Percentage of embryogenic callus from the inflorescences sampled in the years 2020 and 2021 formation in 2020 and 2021.

Production of CAS9, dCAS9-VP64 and CAS12 RR in trasformated DE3 Escherichia coli with pET-28b expression plasmid.

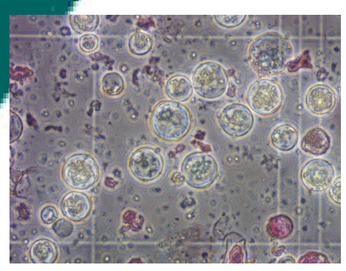


SDS-PAGE analysis of CAS proteins purificated on Ni-NTA agarose followed Sepharose cation exchange resin.



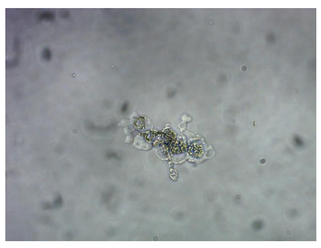
In vitro cleavage test using ribonucleoproteins gRNA/CAS producted and DNA target (VviAGL11-exon 7).

We optimized a protocol for CAS12 production and purification suitable for laboratories not specialized in the production of recombinant proteins. We also produced CAS9 and dCAS9-VP64, a protein lacking the nuclease activity but suitable for activatation of target genes. All produced proteins were *in-vitro* tested to be fully active.

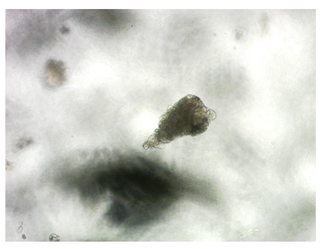


Determination of protoplasts viability with phenosafranin staining (200X).

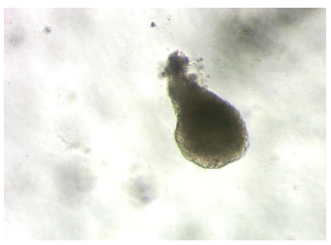
Another step forward was made by improving the protocols to isolate protoplasts and to induce their transfection and regeneration. We tested several protocol and strategies in order to obtain the highest possible yield of viable protoplasts. So far, embryo regenerants from protoplast were obtained.



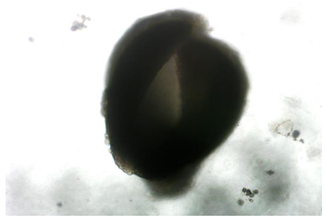
15-day microlony (200X).



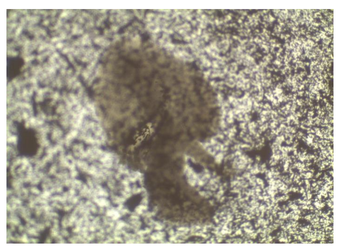
40-day embryo of cv Chardonnay (100X).



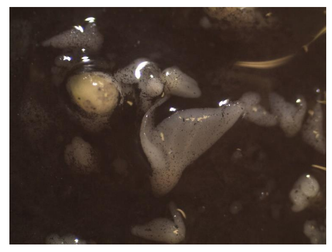
50-day embryo of cv Victoria (100X).



60-day embryo cv Victoria (100X).



70-day embryo cv Chardonnay (20X).



65-day embryo cv Thompson (10X).

Conclusions

The results of this work demontrate that our modified protocols significantly increase the production of embryogenic calluses, exspecially from recalcitrant varieties, and produce viable protoplasts able to regenerate embryos. Regeneration is mandatory for the applicability of NPBTs such as Cisgenesis and Genome Editing. It is necessary to wait at least other 2 to 3 months to verify if the regenerated embryos will result edited.

REFERENCES

Alessandra Amato, Maria Francesca Cardone, Nallatt Ocarez, Fiammetta Alagna, Benedetto Ruperti, Chiara Fattorini, Riccardo Velasco, Nilo Mejía, Sara Zenoni, Carlo Bergamini, VviAGL11 self-regulates and targets hormone- and secondary metabolism-related genes during seed development, Horticulture Research, 2022. https://doi.org/10.1093/hr/uhac133

Forleo, L.R.; D'Amico, M.; Basile, T.; Marsico, A.D.; Cardone, M.F.; Maggiolini, F.A.M.; Velasco, R.; Bergamini, C. Somatic Embryogenesis in Vitis for Genome Editing: Optimization of Protocols for Recalcitrant Genotypes. Horticulturae 2021, 7, 511. https://doi.org/10.3390/ horticulturae7110511

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