PhD School on Agriculture, Environment and Bioenergy

(http://sites.unimi.it/dottorato_aab/)

(XXXIV cycle, 2018-21)

Project draft

1.Field of interest

Settore scientifico disciplinari: AGR 03

2.Project title:

Genetic improvement of grapevine for downy mildew resistance through a cisgenic approach

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- Co-tutor: Barbara De Nardi, CREA

4.Relevance of the topic and state of the art:

The present project is focused on the application of modern biotechnological approaches for the development of downy mildew (caused by *Plasmopara viticola*) resistant grapevine cultivars.

Conventional breeding does not allow the introgression of single traits without compromising the genetic background that characterize an elite cultivar. The exploitation of the new molecular techniques known as genome editing and cisgenesis make possible to modify or transfer single genes preserving all the characteristics selected with difficulty by breeders over a long-time span. To date, 27 QTLs have been associated with downy mildew disease resistance (Rpv1-Rpv27)[1] and many of these have been employed in breeding programs for the introgression in genotypes of interest. Just in case of Rpv1 and Rpv3, however, the underlying genes have been identified and characterized. In both cases nucleotide-binding leucine rich repeat (NB-LRR) genes are present, codifying for receptors that act as cytoplasmic pathogen sensors, triggering a signal transduction pathway for cell-death mediated defense at the infection site. The Rpv1 locus was first identified by bulked segregant analysis in *Muscadinia rotundifolia*[2]. Later *Dry et al.* (2014) designated MrRGA8 gene (14,6 Kb in size on chromosome 12) as Rpv1, showing its nucleocytoplasmic localization and its ability to generate alternatively spliced transcripts[3].

The Rpv3 locus was identified by interval mapping in ‘Regent’ and further characterized in ‘Bianca’ where *Bellin et al.*[4] (2009) used a 116 full-siblings population from the cross ‘Chardonnay’ x ‘Bianca’ to demonstrate that the hypersentive response to the pathogen infection is a Mendelian trait controlled by a major gene. Either ‘Regent’ and ‘Bianca’ descend from ‘Seibel 4614’, one of many hundred hybrids of the series ‘Seibel’ and ‘Seyve Villard’ produced by the French breeders who aimed at introgressing resistances from North American species (*V. aestivalis, V. berlandieri, V. cinerea, lincecumii, and V. rupestris)* into *V. vinifera* background, however the species who originally donated the Rpv3 resistance is unknown. The locus has been mapped on chromosome 18 and is traceable by its association with rare alleles at two microsatellite markers (UDV305 and UDV737) that flank both sides of Rpv3 within an interval of 1.4 cM[1,5]. In this region, two NB-LRR candidate genes, TNL2a and TNL2b, with a length of about 5k bp each as well as a LRR-kinase receptor with size 7k bp [6] are present. TNL2a also undergo through multiple alternative splicing. Alternative transcripts are indeed frequently reported for resistance genes and even if their role is yet to clarify in several documented cases they have been proved to be essential for pathogen restriction.

One of the biggest drawbacks of traditional genetic engineered plants is represented by the presence of transgenes (often selection markers), usually perceived as unsafe by consumers. The cisgenic approach aims at circumventing this problem avoiding the presence of exogenous DNA, introducing only the desired trait by using native genes from *Vitis* species, interfertile with *V. vinifera*. Adopting this strategy, the already characterized resistance genes RPV1 and/or RPV3 will be introduced in some elite *Vitis vinifera* varieties, highly appreciated by the wine industry.
The outcomes will reduce the agrochemicals needs and the risks associated with their use, increasing the profitability of the vineyard and consumers' appreciation.

5.Layout of the project (draft)

5.1.Materials & Methods:

Plant Material

Vegetative buds from selected elite cultivars will be collected and surface sterilized for the obtainment of micropropagated plantlets. For the same genotypes, flower tissues (anthers and ovaries) collected from field grown plants and from fruiting cuttings, will be used as explants for the induction of somatic embryos[7,8].

Vector construction

PCR products of candidate genes including native promoter and terminator will be cloned in a suitable vector (i.e Invitrogen pCR-XL-TOPO vector) and transformed into competent *E. coli*, following the manufacturer's instructions. The gene sequences will be then isolated by PCR and cloned into pMF1[9] analogous binary vector. After verification of the proper configuration and sequences the plasmid will be isolated and trasformed into the *A.tumefaciens* strain of choice.

Transient expression

Transient expression assays will be used to evaluate the efficacy of the candidate genes into the different genetic backgrounds of the selected cultivars. Agroinfiltration of in vitro plantlets by vacuum and agrodrenching for systemic expression will be evaluated.

Transformation and regenerant selection

The gene construct will be used for the transformation of grapevine embryogenic calli through *A. tumefaciens* infection. Infected calli will be transferred on selective media for the induction and germination of somatic embryos. Regenerated plantlets will be checked for the presence and expression of candidate genes by qPCR or sequencing. Absence of *Agrobacterium* and backbone sequences will also be checked by PCR on transformants. For the removal of exogenous sequences, the use of an excision strategy will be evaluated (i.e. removal of T-DNA through recombination induced by heat-shock; dexamethasone treatment coupled to 5-fluorocytosin selection[10]).

Plasmopara viticola resistance assay

Resistance and susceptibility to downy mildew will be tested on available material of interest by leaf disc bioassay or whole leaves inoculation of in-vitro and/or acclimatised plantlets.

5.2.Schedule and major steps (3 years):

To fulfill the aforementioned objectives the work will be structured as follow:

First Year

In the first year the main activities will be focused on the induction of embryogenic calli from flower tissues and the micropropagation of in vitro grapevine plantlets. Design of gene cassette and construction of the vectors for transformation experiments will be performed.

Second Year

During the first part of second year transient assay will be performed, evaluating the infection-efficiency of different *A. tumefaciens* strains. Then stable transformation will be carried out on embryogenic calli of varieties of interest.

Third Year

Transformants will be regenerated and screened for the presence of the desired gene sequences. Possibly, the phenotypic trait (resistance to downy mildew) will be evaluated on leaves or plantlets.

6. Available funds (source and amount)

The above mentioned activities will be supported by Ministerial founds (BIOTECH project, VITECH subproject).

6. Literature:

1. Sapkota, S. *et al.* Construction of a high-density linkage map and QTL detection of downy mildew resistance in Vitis aestivalis-derived 'Norton'. *Theor. Appl. Genet.* **132**, 137–147 (2019).
2. Merdinoglu, D. *et al.* Genetic analysis of downy mildew resistance derived from Muscadinia rotundifolia. Proceedings of the Eighth International Conference on Grape Genetics and Breeding. *Acta Hortic.* **603**, 451–456 (2003).
3. Dry, I. & Mark, T. Molecular and genetic strategies to reduce the susceptibility of wine grapes to fungal pathogens FINAL REPORT to Project Number : CSP 0903 Principal Investigators : Dr Ian Dry & Dr Mark Thomas Research Organisation : CSIRO Plant Industry. (2014).
4. Bellin, D., Peressotti, E. & Merdinoglu, D. Resistance to Plasmopara viticola in grapevine ' Bianca ' is controlled by a major dominant gene causing localised necrosis at the infection site. 163–176 (2009). doi:10.1007/s00122-009-1167-2
5. Di Gaspero, G. *et al.* Selective sweep at the Rpv3 locus during grapevine breeding for downy mildew resistance. *Theor. Appl. Genet.* **124**, 277–286 (2012).
6. Foria, S. & Gaspero, G. Di. Dottorato di Ricerca in Scienze e Biotecnologie Agrarie Coordinatore : prof . Mauro Spanghero The Rpv3 locus in grapevine : DNA variation and relevance for conventional breeding. (2015).
7. Gribaudo, I., Gambino, G. & Vallania, R. Somatic Embryogenesis from Grapevine Anthers : The Optimal Developmental Stage for Collecting Explants. **4**, 427–430 (2004).
8. Gambino, G. & Ruffa, Æ. P. Somatic embryogenesis from whole flowers , anthers and ovaries of grapevine (Vitis spp .). 79–83 (2007). doi:10.1007/s11240-007-9256-x
9. Schaart, J. G., Krens, F. A., Wolters, A.-M. A. & Visser, R. G. F. Transformation Methods for Obtaining Marker-Free Genetically Modified Plants. *Plant Transform. Technol.* 229–242 (2011). doi:10.1002/9780470958988.ch15
10. Krens, F. A. *et al.* Cisgenic apple trees; development, characterization, and performance. *Front. Plant Sci.* **6**, (2015).