“Clean” genome editing in grapevine (*Vitis* ssp.)

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BACKGROUND

In recent years new plant breeding techniques (NPBT), and in particular genome editing via Crispr/*Cas9*, emerged as breakthrough tools for the genetic improvement of agricultural species, allowing to precisely modify specific genes in shorter time compared to traditional breeding and without altering the genetic heritage of cultivars. Grapevine may receive a major benefit from NPBT being viticulture production based on a few elite cultivars.

To date the European Commission (EC) has not yet deliberated on the legal status of NPBT products, whether they should or should not be covered by GMO legislation. A technical aspect that could be considered to reach a decision is the presence or absence of exogenous DNA in the plant genome (e.g. the genetic elements of the editing machinery) and, in case of remaining traces, their lenght.

KEY MESSAGE: We applied the **Crispr/Cas9** system in **grapevine** for the inactivation of the ***VvMLO7* gene** which plays a key role in susceptibility to **powdery mildew**.

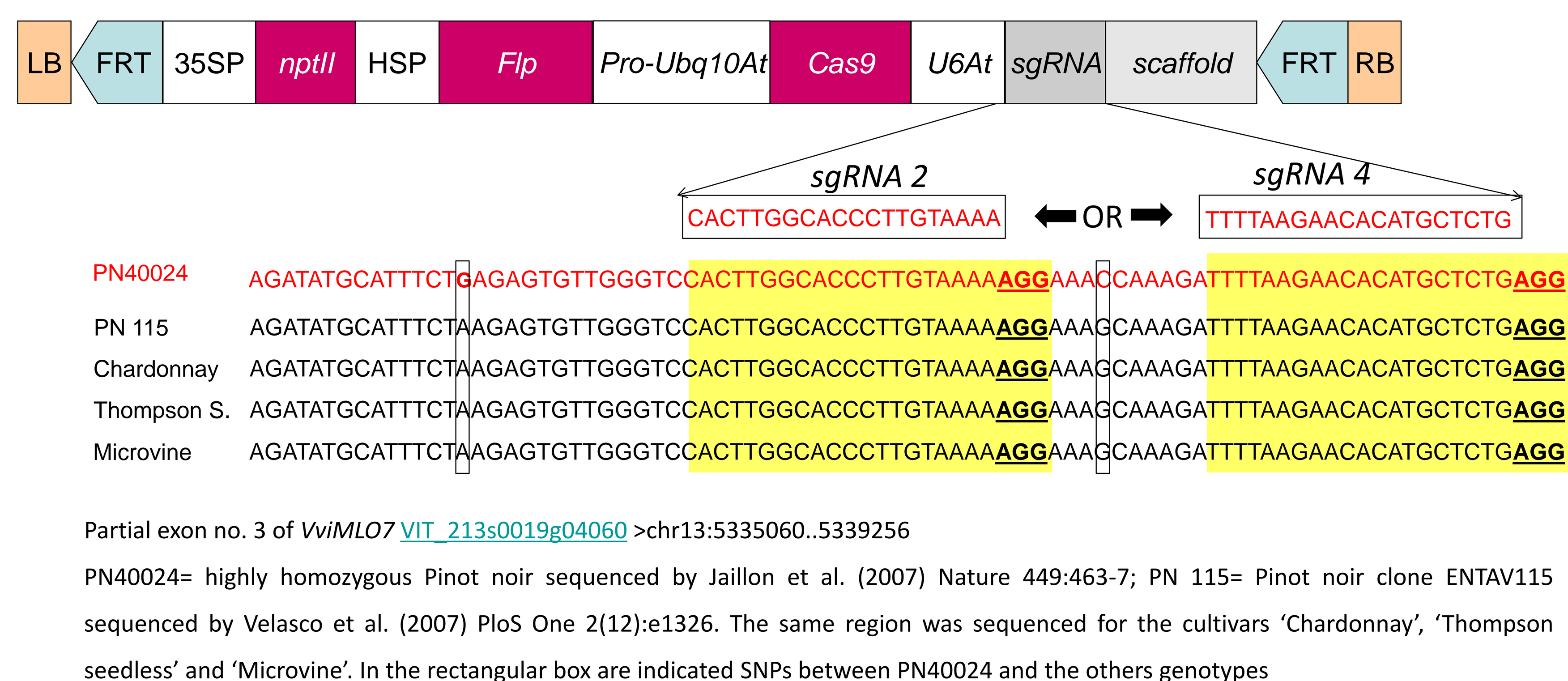
The novelty of our “**clean**” strategy is that it aims at leaving in the plant genome the **minimal trace of exogenous** DNA.

METHODS and PRELIMINARY RESULTS

1 DESIGN OF THE VECTOR :

The binary vector contains the *Cas9* gene, the sgRNA and the selection marker gene *nptII*. Moreover, the T-DNA cassette may be removed by exploiting the *Flp* recombinase gene (under the control of an heat-shock inducible promoter HSP) and its recognition sites (FRT), placed right next to the *A.t.* left and right borders.

Due to the high genetic variation among grapevine cultivars and accessions, the design of the sgRNA requires the sequencing of the target regions in the genotypes of interest to ensure the absence of undesired SNP.



Partial exon no. 3 of *VviMLO7* VIT_213s0019g04060 >chr13:5335060..5339256

PN40024= highly homozygous Pinot noir sequenced by Jaillon et al. (2007) Nature 449:463-7; PN 115= Pinot noir clone ENTAV115 sequenced by Velasco et al. (2007) PloS One 2(12):e1326. The same region was sequenced for the cultivars ‘Chardonnay’, ‘Thompson seedless’ and ‘Microvine’. In the rectangular box are indicated SNPs between PN40024 and the others genotypes

2 GENE TRANSFER:

The classical *Agrobacterium tumefaciens* (*A.t.*) was used to transfer the T-DNA cassette in embryogenic calli of ‘Chardonnay’, ‘Thompson seedless’ and ‘Microvine’. The selection phase was carried out in the presence of kanamicine 150 mg/L. After 6-9 months developed embryos at the cotiledonary stage were transferred in germination medium at light and with a lower dose of kanamicine. Some of them regenerated a plantlet.

‘Chardonnay’ ‘Thompson seedless’ ‘Microvine’

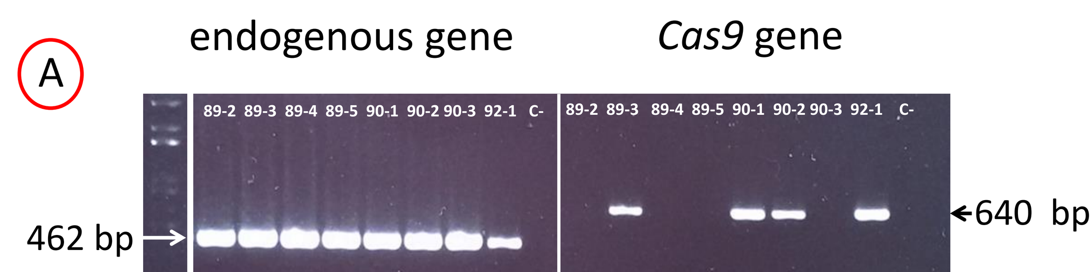
3 MOLECULAR CHARACTERIZATION

(A) Screening with a qualitative PCR for the amplification of Cas9 gene

(B) Determination of *nptII* CN with a quantitative Real-time PCR

(C) Determination of the occurring mutation in the target site by Illumina sequencing

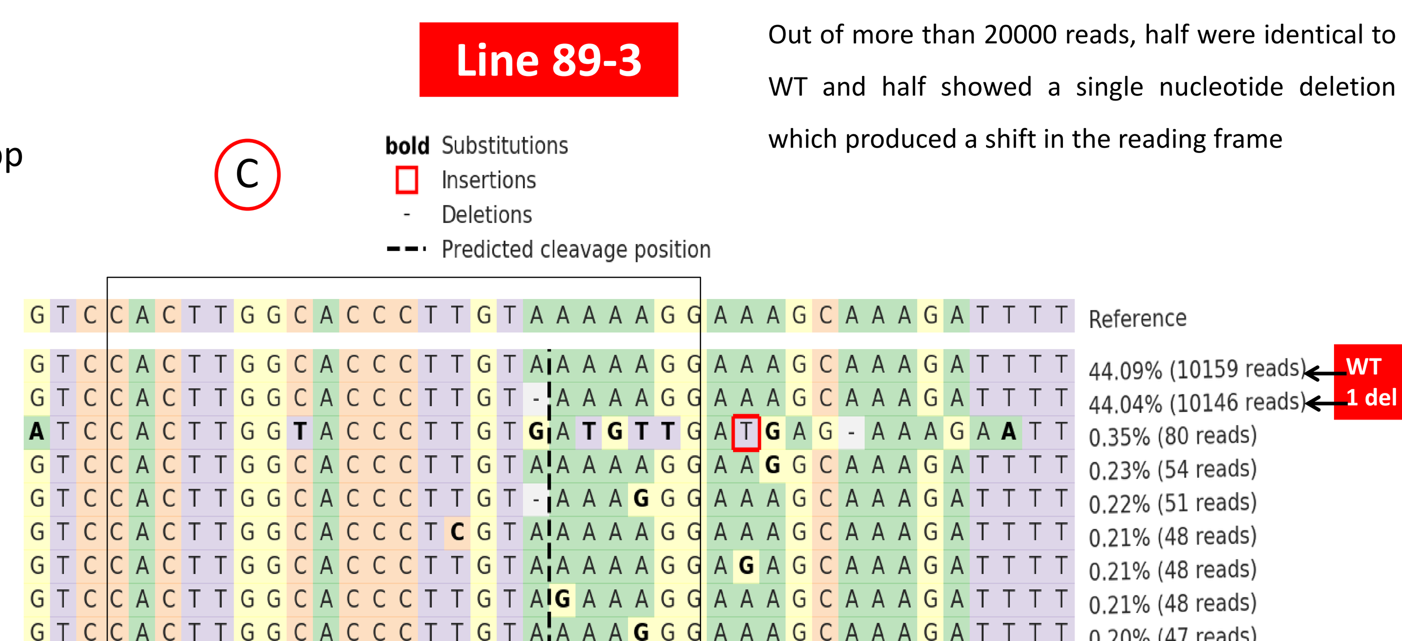
(A) endogenous gene — *Cas9* gene



(B)

Line ID	genotype	sgRNA	*nptII* CN
89-3	Chardonnay	sgRNA 2	0.71 ± 0.22
90-1	Thompson s.	sgRNA 2	0.92 ± 0.06
90-2	Thompson s.	sgRNA 2	0.94
90-4	Thompson s.	sgRNA 2	0.83 ± 0.18
92-1	Microvine	sgRNA2 + sgRNA4	1.88

(C)

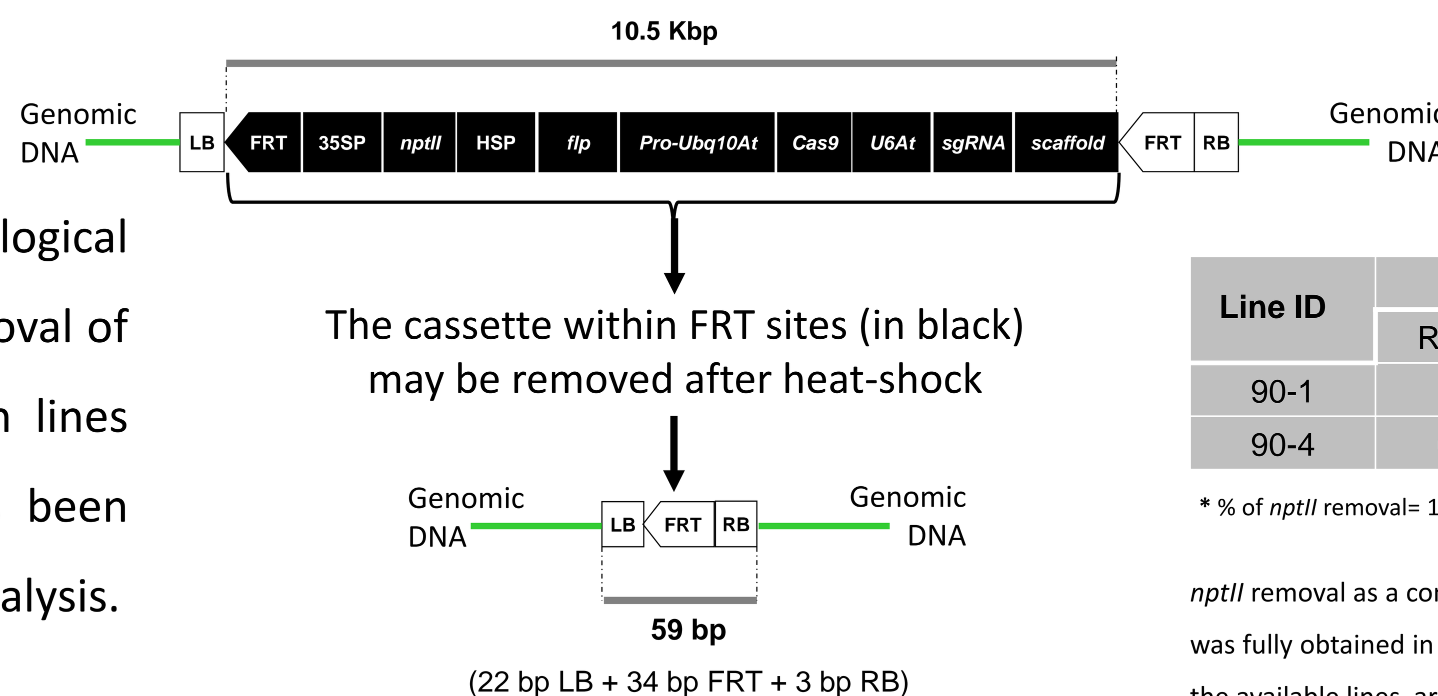


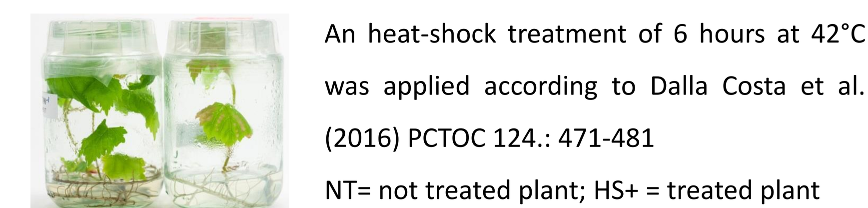
Out of more than 20000 reads, half were identical to WT and half showed a single nucleotide deletion which produced a shift in the reading frame

WORK IN PROGRESS

1 T-DNA CASSETTE REMOVAL

An heat-shock treatment was applied to biological replicates of lines 90-1 and 90-4 to induce the removal of the T-DNA contained within FRT sites. Thompson lines showed severe leaf tissues damages but it has been possible to collect some green tissue for *nptII* CN analysis.





An heat-shock treatment of 6 hours at 42°C was applied according to Dalla Costa et al. (2016) PCTOC 124.: 471-481

NT= not treated plant; HS+ = treated plant

Line ID	*nptII* CN				% of *nptII* removal *
	Repl. 1	Repl. 2	Repl. 3	Mean	
90-1	1.08	0.97	0.90	0.98 ± 0.09	0 %
90-4	0.89	0.82	0.00	0.57 ± 0.49	31 %

* % of *nptII* removal= 100 – (*nptII* mean CN of treated sample / *nptII* mean CN of untreated sample x 100)

nptII removal as a consequence of the cassette excision was not achieved in lines 90-1 while it was fully obtained in replicate n. 3 of line 90-4. New and repeated rounds of heat-shock on all the available lines are in progress

2 IN VIVO PATHOGENICITY ASSAY

The susceptibility to *Erysiphe necator,* the causal agent of powdery mildew, is going to be tested in the grapevine line 89-3 where the functional form of *VvMLO7* is available at 50%. Biological replicates of line 89-3 and Chardonnay WT were acclimatized in greenhouse and will be soon inoculated with the fungus .

CONCLUSION

Thanks to the huge and continuous technological progress, a likely perspective is that in the future improved plants with desired traits will be produced with the minimal and “cleanest” genetic modification and advanced analytical tool will be available for their deep and thorough characterization and evaluation.