Proceedings of the LXIV SIGA Annual Congress
Online, 14/16 September, 2021
ISBN: **978-88-944843-2-8**

Oral Communication Abstract – 4.05

GENOME EDITING APPLIED TO INDUCE LYCOPENE ACCUMULATION IN ANTHOCYANIN-RICH SWEET ORANGE VARIETIES

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CRISPR/Cas9, GoldenBraid 3.0, antioxidant compounds, fruits, Citrus

New plants breeding techniques (NPBTs) represent modern biotechnological approaches used to improve plant varieties, generally traditional and of high-value accessions, already excellent except for one or few traits that penalize the product. One of the advantages of NPBTs consists in overcome the limits of traditional breeding. Application of NPBTs depends on the knowledge of those genes controlling specific traits; however, in citrus only few genes are well characterized. Also, reliable transformation protocols are needed for each specific variety being some of them recalcitrant to regeneration. In the last years, citrus breeding programs have been also focused on the obtainment of varieties producing fruits with enhanced content of bioactive compounds, such as anthocyanins and lycopene (flavonoids and carotenoids, respectively), deeply studied for their beneficial effects on several human diseases. However, to date, no citrus varieties are reported to bear fruits highly enriched in both pigments.

In this work, we present a genome editing approach to induce the knockout of beta-cyclase 2 (*β-LCY2*) gene on an anthocyanin-rich sweet orange variety (Doppio sanguigno, DS) and on Carrizo citrange (CC), as model species. Previous studies showed a low expression of β-LCY2 in lycopene-rich citrus mutants, compared to common varieties. The design of two sgRNAs, spaced 250 bp, was approached to create a potential large deletion or to lead punctual mutations in one or both sgRNAs. Genome editing vector was realized following the cloning strategy of GoldenBraid 3.0; it includes the two

sgRNAs, and the cassettes for *Cas9* and for *nptII* genes. The plasmid was introduced in EHA105 strain of *Agrobacterium tumefaciens* and used for transformation, which were performed using internodal stem segments.

After cocultivation and regeneration onto selection media, resistant shoots of CC and DS were obtained and validated by PCR to detect the presence of *Cas9* and *nptII* genes. So far, 149 CC shoots C*as9-and-nptII* PCR positive have been self-rooted, while 66 DS shoots *Cas9-and-nptII* PCR positive were mini-grafted onto Carrizo seedlings.

Actually we amplified through PCRs (which primers have been designed to include both sgRNAs on the *β-LCY2* gene) 30 plantlets for each genotype, CC and DS. Overall, we observed three different profiles, and in particular: 2 plantlets of CC and 4 of DS showed two bands, one of 106 bp (corresponding to the deletion of 271bp, that is the distance between primers upstream and downstream both guides) and one of 380 bp (assuming that single mutations occurred in one or both sgRNAs); other 2 of CC showed a single band of 106 bp; all the remaining plantlets reported a single band at 380 bp. Illumina sequencing, currently in progress, will elucidate which kind of mutation has occurred on each sgRNAs.

This study represents the first attempt to use CRISPR/Cas9 genome editing approach to improve qualitative traits in citrus varieties.